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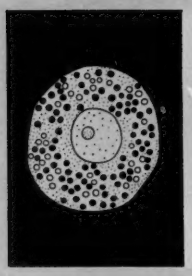

ACHIEVEMENTS IN SOVIET MICROBIOLOGY

Edited by A. A. Imshenetskii

Director of the Institute of Microbiology of the Academy of Sciences of the USSR

TRANSLATED FROM RUSSIAN

(Original published for the Institute of Microbiology, Academy of Sciences of the USSR, by the Academy of Sciences Press, Moscow)

This concise survey of recent developments in (as well as projected directions of) Soviet microbiology is divided into four major sections:

I. Progress in General Microbiology, *A. A. Imshenetskii*
A broad review which enables the Western scientist to see clearly the direction in which Soviet microbiologists are making their studies.

II. Achievements of Soviet Microbiology in the Field of the Study of the Geological Activity of Microorganisms, *S. I. Kuznetsov*

In a field which has not been widely considered by Western scientists, this paper indicates great advances made in the USSR. The use of microorganisms in petroleum research and recovery are discussed, as are Soviet efforts to classify precisely and map out geologic or ecologic microbiological strata as they occur in the geologist's scheme. This article summarizes Soviet research into the relationships of various groups of microorganisms to geological – petroleum – inorganic substrates environments.

III. Forty Years of Technical Microbiology in the USSR, *V. N. Shaposhnikov, N. D. Ierusalimskii, and I. L. Rabotnova*

Noteworthy in this paper (which deals with fermentation microbiology, acetone-butanol, organic acids, riboflavin, antibiotics, etc.) is Shaposhnikov's description of disagreement in the USSR, as there has been in the West, on the biosynthesis of citric acid by various species of *Aspergillis.*

IV. Achievements in the Field of Soil Microbiology in the USSR, *E. N. Mishustin*

Soviet research in this area places particular emphasis on rhizosphere, root nodule, and nitrogen-fixing bacteria, and on the relationship between higher plants and associated microorganisms. Special attention is given to Vinogradskii's early description of auxotrophs and Krasilnikov's recent classification of actinomycetous microorganisms. Some comment is made on methodology, especially microbial counts and selection techniques.

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BIOCHEMISTRY

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Vol. 26, No. 2 pp. 187-348 September-October, 1961

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ERRATA

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Page	Column	Line
324	Right	8-11

Should read:

In measuring the adsorption of the protein fractions (serum albumin) we prepared 5 to 7 solutions of different fractions. Serum albumin solutions (with the methionine fraction preparations) were denatured by the method described....

Page	Line
308	20, 19 from bottom

Should read:

after irradiation it was equal to 19 ± 2.23 cpm/mg. Analogous data were obtained for liver from females.

PROTEIN CHROMATOGRAPHY ON CALCIUM PHOSPHATE GEL

E. S. Zueva, L. S. Markosyan and N. I. Proskuryakov

Chairman of the Plant Biochemistry of M. V. Lomonosov State University, and
A. N. Bakh Institute of Biochemistry, Academy of Sciences, USSR
Translated from Biokhimiya, Vol. 26, No. 2,
pp. 209-211, February, 1961
Original article submitted April 15,1960

One of the means of purification and fractionation of proteins is adsorption chromatography on calcium phosphate columns after Tiselius [1]. Thanks to its simplicity and availability, this method now begins to be widely used. By this method, Tiselius was able to fractionate a whole series of proteins: serum albumin, lysozyme, γ-globulins, etc. By using the same procedure, Johansson [2] fractionated lactalbumin. An analogous method has been also by others [3,4] for the purification of some enzymes. This method could be also applied to the study of nucleic acids [5,6].

In experiments on adsorption chromatography of proteins on calcium phosphate columns, one must take into account the possibility of obtaining artifacts when using buffer solutions with very small differences in concentration. It is not excluded, also, that protein substances of different nature may appear simultaneously and jointly in some particular fractions. It follows that one can assess the real individuality and homogeneity of a protein revealed in a particular peak only after repeated chromatography of the given fraction in buffer solutions of required concentrations.

EXPERIMENTAL

For the study of protein preparations of different origin, we used 1.5 x 15 cm columns placed inside glass jackets with side tubes, through which water at 4-5° was circulated. The columns were filled with the adsorbent without applying pressure. As adsorbent we used hydroxyapatite gel [$Ca_5(PO_4)_3OH$], prepared according to Tiselius [1], and kept until it was needed in a 0.001 M phosphate buffer at pH 6.8. Elution was carried out with buffer solutions of gradually increasing concentrations. The eluent was passed at the rate of 3 ml/20-40 min. The protein was estimated spectrophotometrically at 280 mμ.

First, we repeated Tiselius' experiments on the fractionation of egg albumin, and our results were sufficently close to his.

A preparation of human serum albumin was investigated by the same method. The given preparation was first submitted to electrophoresis in a Tiselius-Svenson apparatus by the method of moving boundary (see the table).

On the basis of the results obtained, one can consider that under the given experimental conditions, the preparation of serum albumin behaves as a homogeneous protein; investigation of the preparation by paper electrophoresis led to the same conclusion. On the other hand, gradient fractionation on a calcium phosphate column gave three different albumin fractions. The first fraction, eluted with 0.07 M phosphate buffer at pH 6.8, contained 61.92% of albumin; the second fraction, eluted with 0.11 M buffer – 27.98%, and the third fraction, eluted with 0.4 M buffer – 10.1%. The total amount of albumin in the eluate was almost the same as the starting quantity. These data prove, therefore, the heterogeneity of serum albumin.

Especially interesting and promising was the investigation along the same lines of a preparation of water-soluble protein, obtained from wheat seeds of "Erinatium" variety by the method of Pence and Elder [7], used

Electrophoretic Mobility of Human Serum Albumin and Wheat Flour Albumin Preparations. Drop of tension, 4 v/cm; veronal buffer, at pH 8.6 for serum albumin, and 8.1 for wheat flour albumin; ionic strength, 0.1; temp., 1°

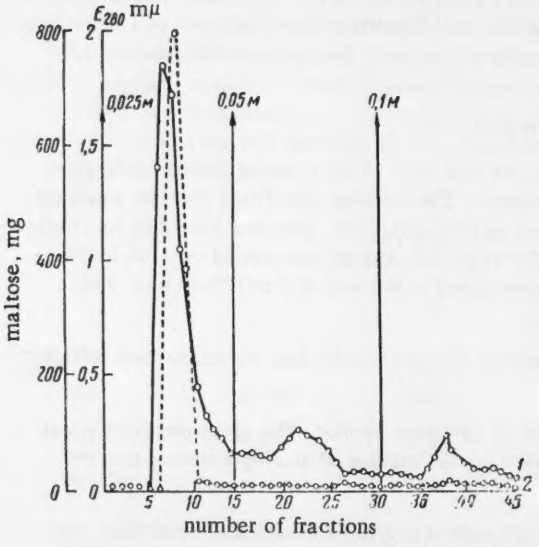
Albumin	Electrophoretic mobility of the components* (10^{-5} $cm^2 \times v^{-1} \times sec^{-1}$)				Albumin concentration in %
	I	II	III	IV	
Serum albumin	5.15	–	–	–	1.0
Wheat flour albumin	4.3	3.0	2.6	0.8	1.2

* The magnitudes of mobilities were obtained by taking the mean of the boundary data in the ascending and descending sections.

with some modifications [8]. The mentioned preparation contained 12.5% of nitrogen, about 10% of carbohydrates, and about 6% of ash. As shown by Proskuryakov and Khromova [9], and by Markosyan [8], such preparations of wheat flour albumin contain some enzymes, viz., amylase, phytase and polyphenol oxidase. As there was a possibility of obtaining separate protein fractions enriched in the one or the other of the mentioned enzymes, it was interesting to carry out the fractionation of these albumin preparations.

The preparation of wheat flour albumin under investigation was also submitted to a preliminary study in the Tiselius-Svenson apparatus; the data obtained are shown in the table. From the given results it follows, that under the experimental conditions used, the wheat flour albumin is fractionated to four components which considerably differ from one another in electrophoretic mobility.

The stepwise fractionation of the given preparation was carried out as follows. The albumin preparation (50 mg) was dissolved in 2 ml of 0.005 M buffer at pH 6.8, and the solution was placed on the top of the column. Elution was carried out first with a 0.025 M buffer, followed by a 0.05 M buffer, and finally, with a 0.1 M buffer at the same pH, the volume of each buffer being 45 ml. The eluate was collected in 3 ml fractions. Each of the 45 fractions thus obtained was assayed for albumin and amylase activity, the latter being determined by its effect on soluble starch in 15 min at 37°, with the estimation of maltose according to Somogyi [10]. Calculation showed that the total amount of albumin in the collected fractions was exactly the same as the initial amount taken. The first 15 fractions contained 82.38% of albumin, the following 15 fractions – 11.26%, and the last fractions – 5.92%. The same results concerning the distribution of albumin in different fractions were observed when the experiment was repeated under the same conditions.



Distribution of albumin and amylase activity between different fractions during elution with buffer solutions of different concentrations. 1) albumin content in E units at 280 mμ; 2) amylase activity in mg of maltose per 3 ml of eluate.

Data pertaining to the quantitative estimation of albumin and amylase activity in all the fractions are represented in the figure. All the amylase activity was concentrated in a very small number of separate fractions, and was completely missing in the others. Thus, the amylase activity, calculated on the basis of 1 mg of albumin, was 16.5 times higher in the seventh fraction than in the starting material. Therefore, the application of the given method of adsorption chromatography has allowed to concentrate, and, to a certain degree, to purify the albumin component of the preparation possessing an amylase activity. Application of the described method may render possible the separation of highly purified enzyme preparations during the process of their isolation.

SUMMARY

Fractionation of several protein preparations was carried out on calcium phosphate gel columns by stepwise elution with phosphate buffer of gradually increasing concentration.

It was shown that β-amylase activity characteristic of the preparation of the water soluble protein fraction of wheat flour was confined only to a few fractions, completely absent in others.

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CHANGES IN MINERAL METABOLISM AND NITROGEN CONTENT OF RAT BONE AS A FUNCTION OF AGE

H. I. Elkina

Translated from Biokhimiya Vol. 26, No. 2, pp. 212-215
February, 1961
Original article submitted April 26, 1960

The literature contains some results about the effect of age on chemical composition and metabolic processes in bones of man and animals [1-9]. In most cases the investigations were carried out without separating bone into its epiphysial and diaphysial fractions and were restricted to a rather short period of the animal's life. However, studies carried out on separate age groups of animals showed that the epiphysial and diaphysial fractions of bone differ not only as to the rate of metabolic processes but also as to the content of minerals and nitrogenous compound [3,5,10-12].

In this connection, it appeared of interest to make a more complete study of mineral metabolism and nitrogen content in the individual epiphysial and diaphysial fractions of tubular bone over a more prolonged period of the animal's life than was studied in earlier investigations.

METHODS

Experiments were carried out on 83 male white rats from 30 to 630 days old. The animals received a diet containing 0.43% calcium, 0.44% phosphorus, and 0.2 g of cod-liver oil. After the rats had been killed by exsanguination, the fore- and hindlimbs were freed from their articulations as quickly as possible, and the bones were carefully cleaned of muscles and connective tissue. Because of the small quantity of tissue available for analysis, bones of five animals at 30 days of age were pooled, and two rats were pooled at 75 days (all treatment of the tissue was carried out in the cold). The epiphysial (epiphysis + metaphysis) and diaphysial (diaphysis) fractions were rinsed clean of bone marrow by two washes in physiological solution and one wash with distilled water, while the bones were simultaneously being cut up with shears, for 5-7 minutes. The cleaned and minced bones were analyzed for content of Ca, P, Mg, Sr, total N, rate of incorporation of Ca^{45} and P^{32}, and activity of alkaline and acid phosphatase. Calcium was analyzed by the permanganate method of Fiske and Subbarow [13], and nitrogen by the Kjeldahl method.

Mg and Sr were analyzed spectrally by the method of three standards [14] after the bones had first been dissolved in HNO_3. Ni was used as an internal standard for the standard solutions and unknowns. Spectrophotometry

Content of Calcium, Phosphorus, Magnesium, Strontium, and Total Nitrogen in Rat Bones

Age of animals, days	Weight g	Number of animals	Ca		P		Mg
			I	II	I	II	I
30	42± 1,2	25	265,0±12,8	209,0± 5,9	163,0± 5,2	123,0±8,1	5,20±0,30
75	120± 1,6	10	296,0±15,6	253,0±12,0	151,0± 4,8	125,0± 5,2	—
180	258± 7,6	12	307,0± 8,2	243,0± 6,6	148,0±11,6	119,0±9,5	—
270	290±10,8	9	324,0± 7,6	271,0±13,4	130,0± 4,7	119,0±2,5	3,30±0,10
440	371±11,8	15	301,0±10,6	278,0± 9,3	139,0± 5,6	125,0±3,3	—
630	373±18,5	12	327,0± 6,4	282,0± 5,5	157,0± 3,0	132,0±2,7	3,40±0,05

Symbols: I) diaphysis; II) epiphysis.

was carried out on the ISP-22 spectrophotometer. The source of excitation was the AC arc PS-39, at a current strength of 5.5 amp. The lines used for analysis were: Mg 2779.8 A; Ni 2798.7 A; Sr 4077.7 A; Ni 3722.5 A.

To study the rate of incorporation of Ca^{45} and P^{32}, animals were injected intraperitoneally with a solution of a radioactive mixture (at pH 5), computed on the basis of 0.1 μC P^{32} and 0.05 μC Ca^{45} per g body weight, 24 hours before the animal was sacrificed. The quantity of Ca and P in the radioactive mixture was very small and could not have affected the content of these elements in bone tissue. Activity due to Ca^{45} and P^{32} was measured by a type B counter, which differentiates between their radiation by aluminum filters. To compare the rate of incorporation of Ca^{45} and P^{32} into bone tissue of different age groups of animals, the specific activity (S.A.) was computed by the following formula:

$$\text{S.A.P (Ca)} = \frac{\text{counts/min/g bone} \times 100}{\text{counts/min/g body weight} \times \text{mg P (Ca)/g bone}}$$

For studies of phosphatase activity, the surce of the enzymes was an aqueous extract of bone tissue, prepared by the method of Cohn and Gong [8]. Na β -glycerophosphate was used as substrate. Activity of alkaline phosphatase was estimated at pH 8.7, acid phosphatase at pH 4.9. Incubation was carried out for 24 hours at 37°. All results in this paper were computed per g dry tissue and expressed as mean values and mean square errors. Comparison of mean values for separate groups was made by computing the error of the difference.

EXPERIMENTAL

The results of estimates of Ca, P, Mg, Sr, and total N content are set out in the table. All differences to be discussed in this paper are statistically significant. As apparent from the table, the diaphysis contains 16-26% more Ca than the epiphysis, and 10-32% more P. As for changes in mineral composition of bone during growth, it should be noted that the Ca content increased with growth both in the epiphysial and diaphysial fractions.

During the period under study the quantity of Ca increased by 23% in the diaphysis and by 33% in the epiphysis. The P content of both bone fractions hardly increased over this period of growth. By 270 days a slight decrease in the P content of the diaphysis was in fact noticeable. It is interesting that other investigators [3,9] also observed a drop in the P content of bone at approximately this time as compared with previous experimental periods. Beyond this period some increase of the P content occurred in both the diaphysial and epiphysial fractions.

Sr and Mg analyses showed no noticeable difference in the content of these elements in different fractions of tubular bone. As the age of the animals increased from 30 to 270 days, the Sr content increased by 20% in the diaphysis and by 36% in the epiphysis. In the last period studied the Sr content underwent no change. The quantity of Mg decreased somewhat as the rats aged. The content of total N was 30-70% higher in the epiphysis than in the diaphysis; in young rats these differences were even more striking. The content of total N in bone tissue of rats dropped significantly with age. Thus, between 30 and 75 days the content of total N decreased by 22% in the diaphysial fraction and by 31% in the epiphysial fraction. The greatest decline was observed towards the 270th day of life. In subsequent experimental periods the nitrogen content remained at the same level. Tarjan and Szoke [9] observed similar tendencies in the changes of Ca, P, and N content in tubular bones of rats as the animals grew from 4 weeks to 42 weeks old.

as a Function of Age (mg/g dry tissue)

	Sr		N		Ca/N		P/N	
II	I	II	I	II	I	II	I	II
4,10±1,20	0,150±0,010	0,140±0,020	52,3±1,9	88,7±6,4	5,10	2,44	3,14	1,41
—	—	—	40,6±1,2	61,7±4,1	7,24	4,16	3,73	2,05
—	—	—	39,0±0,6	50,9±1,5	7,87	4,77	3,79	2,34
3,60±0,20	0,180±0,005	0,190±0,008	33,8±0,6	46,9±1,1	9,59	5,83	3,85	2,53
—	—	—	32,9±0,7	45,8±0,9	9,10	5,94	4,25	2,70
3,20±0,08	0,150±0,005	0,120±0,010	35,1±0 8	48,2±0,7	9,30	5,85	4,52	2,70

To compare the extent of mineralization of bones in different age groups, we computed the ratio of Ca to total N, and also of P to total N. The results showed that the Ca/N and P/N ratios increased with age, and that these increases were greater for the diaphysis than for the epiphysis. The Ca/N ratio attained its highest value in bones of rats 270 days old. Subsequently the Ca/N ratio remained practically unchanged. In studies of the Ca/N ratio in tubular bones of man [6] and animals [9] it was also observed that the Ca/N ratio increased with increasing age, attaining its maximum value in human bones at an age of 20-30 years, when epiphysial growth is virtually complete. The P/N ratio increased continuously throughout the whole period under investigation.

Study of Ca and P metabolism by means of radioactive tracers showed that their S.A. was higher in the epiphysis than in the diaphysis (Fig. 1). In young, rapidly growing rats the rate of incorporation of Ca^{45} and P^{32} was significantly higher than in old animals. Thus, the S. A. of Ca and P during the period from 30 to 270 days decreased by 55% and 65%, respectively. In the bones of old rats the tendency towards further decreases was less pronounced. By 630 days the S.A. of Ca in bones was 28% of its value at 30 days, while the corresponding figure for the S.A. of P was 7%. The uninterrupted decline in the rate of incorporation of Ca^{45} and P^{32} as the animals grow older was correlated with a decrease in phosphatase activity.

The activity of alkaline phosphatase (Fig. 2) decreased on the average by 76% over the course of the animals' life from 30 to 270 days. In the subsequent periods studied, almost no further decline in the activity of the enzyme was observed. Changes in activity of acid phosphatase with increasing age showed the same trend. For

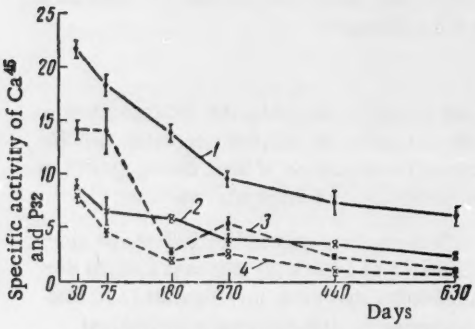

Fig. 1. Change in specific activity of Ca and P in the epiphysial and diaphysial fractions of tubular bones of rats as a function of age. 1,2) Specific activity of Ca; 3,4) specific activity of P; 1,3) epiphysis; 2,4) diaphysis.

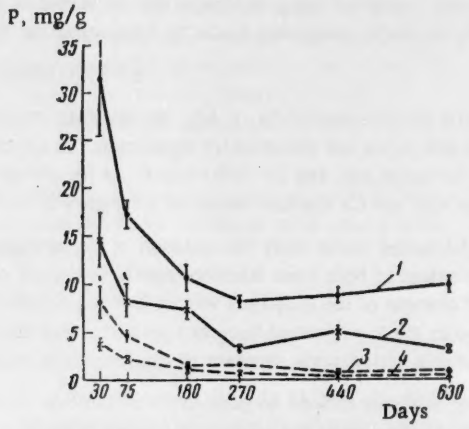

Fig. 2. Change in activity of alkaline and acid phosphatase in the epiphysial and diaphysial fractions of tubular bones of rats as a function of age. 1,2) Alkaline phosphatase; 3,4) acid phosphatase; 1,3) epiphysis; 2,4) diaphysis.

example, in the bones of young rats, in which growth processes are most rapid, the level of enzymes is significantly higher than in old rats. The results support data at hand concerning the important role of bone phosphatase in processes of formation of bone tissue during the growth of the organisms [4,8,15,16].

Comparison of results obtained from studying rats at different ages showed that the most striking changes in composition of bone tissue and rate of metabolic processes were observed during the period of the life of the rats from 30 to 270 days, with maximum values in the period from 30 to 75 days; but variations in the bones of older animals were very small.

SUMMARY

The content of calcium, total phosphorus, magnesium, strontium, total nitrogen, activity of alkaline and acid phosphatases, and rate of incorporation of Ca^{45} and P^{32} into the epiphysial and diaphysial fractions of tubular bones of rats were studied up to the 630th day of life.

During the period from 30 to 630 days the content of Ca increased by 23% in the diaphysis and by 33% in the epiphysis, while the content of N decreased by 33 and 46%, respectively. As the animals grew from 30 to 270

days, the content of P decreased somewhat, but increased between 270 and 630 days. The specific activity of Ca in both fractions of bone decreased by 72%, on the average, by the 630th day, and by 93% for P, as compared to the figures for animals 30 days old. The activity of alkaline and acid phosphatases decreased by 75 and 87%, respectively, from 30th to the 630th day of life.

The greatest changes in composition and rate of metabolic processes of bone tissues were observed during the period of life of the animals from 30 to 270 days. The quantity of total nitrogen, activity of enzymes, and rate of incorporation of Ca^{45} and P^{32} were higher in the epiphysis than in the diaphysis, while the content of calcium and phosphorus was lower in all age groups.

I should like to express my profound gratitude to R. E. Libinzon for guiding this work, and to N. I. Andreeva for carrying out the spectral analyses.

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THE POSSIBILITY OF OBTAINING ELECTROPHORETICALLY HOMOGENEOUS FRACTIONS OF SERUM PROTEINS BY THE METHOD OF SALT PARTITION

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At the present time salt and electrophoretic methods compete in the preparative separation of proteins; at the same time the opinion is strongly expressed in the literature that salt fractions are not identical electrophoretically [1,2]. Preparative electrophoresis permits obtaining separate protein fractions which are homogeneous under conditions of similar control but can be separated into fractions by the salt method [3]. The salt fractions under electrophoretic control are also nonhomogeneous.

The method of salt fractionation with $(NH_4)_2SO_4$, suggested by Hofmeister [4], permits isolation of three protein fractions from blood serum: euglobulin, pseudoglobulin, and albumin. Analogous fractions were obtained by Howe [5] and others using Na_2SO_4. Cohen and co-workers [6] and Svensson [7] improved the method of salt partition; by strict control of the pH they succeeded, using $(NH_4)_2SO_4$, in separating serum proteins into five fractions. Here the fractions obtained by electrophoresis were shown to be nonhomogeneous.

Fractionation of blood serum proteins has also been carried out with salts of heavy metals, organic compounds, and solvents. Thus, Gorzheisha and Smetana [8], using rivanol, isolated γ-globulin of a high degree of purity from blood serum. Lewer and co-workers [9] used zinc salts for preparative separation of plasma proteins and as a result of a multi-stage process they were able to isolate γ-globulin. For the precipitation and analytical determination of γ-globulin Kunkel [10] also used zinc ions.

Cohn and co-workers [11] and Oncley and co-workers [12] worked out several methods for fractionation of plasma proteins by ethanol. The most useful of these methods were methods 6 and 9 by which they could isolate γ-globulin and preparations which contained the β-globulin fraction. By the method of Cohn and Onclay pure γ_1- and γ_2-globulins were isolated from hyperimmune beef and horse plasma [13]. Pillemer and Hutchinson [14] suggested an analogous but somewhat improved method for separation of plasma proteins using methanol. Keckwick and co-workers [15] using ethyl ether isolated fibrinogen and γ-globulin.

Preparative electrophoresis is also widely used for fractionation of serum proteins. Blix and co-workers [16] first carried out an electrophoretic isolation of γ-globulin from plasma. Cann and Kirkwood [17] and Cann and co-workers [18] by electrophoretic convection obtained γ-globulin from beef serum, and they succeeded in separating the latter into eight fractions. Electrophoresis on a starch block and other immobilizing media permitted separation of separate electrophoretic fractions. However, according to our observations [19], this method produces some qualitative changes and does not permit isolation of the proteins in useful amounts. We obtained an electrophoretically homogeneous fraction of globulin antitoxin from an antidiphtheria serum by combining the methods of salt precipitation and zonal electrophoresis [20] since direct preparation of a homogeneous sample from this serum by electrophoresis was found to be a difficult problem. Here it was shown that with attainment of electrophoretic homogeneity the concentration of antitoxin in the fraction was scarcely increased, and in some cases was even lessened, which indicated the presence of a partial denaturation of the protein under the conditions of the electrophoresis. The resulting electrophoretically homogeneous fraction was nonhomogeneous by the salt

method and by this method could be separated into several subfractions which differed in immune and immunochemical properties, and partly in content of precipitating antibody [21]. In the same way, many crystalline proteins have been shown to be heterogeneous by careful investigation. Thus, crystalline beef serum albumin is complex, consisting of two protein individuals [17], crystalline pepsin has 4-5 components [22] insulin two [23], etc.

In the present study we are attempting to find those conditions of salt fractionation which will assure the possibility of more complete homogeneity in the isolated fractions and the absence of denaturative changes in them.

METHODS

The work was carried out on antidiphtheria horse serum. In the preliminary experiments for preparative separation of the proteins we used $(NH_4)_2SO_4$, Na_2SO_4, $ZnSO_4$, K_2HPO_4 and other salts. We showed that each of them with gradually increasing concentrations and definite pH precipitated a fraction varying in impurity [24,25]. The precipitating agent which most closely corresponded to the above requirements was Na_2SO_4. This salt assured separation of the serum protein into albumin, α, β, and γ-globulins [26-29] without causing denaturative changes even under conditions or repeated reprecipitations which are needed for obtaining a homogeneous fraction. This precipitating agent has the advantage that it does not contain nitrogen, so that it is possible to calculate the amount of protein in the isolated fractions by the Kjeldahl method. For salting out the fractions we used the anhydrous salt, first heated to constant weight in a drying oven at 70-80°.

Course of analysis. One hundred ml of blood serum was diluted with 400 ml of phosphate-borate buffer, pH 6.8 (0.1 M KH_2PO_4 and 0.2 M borax). The serum solution was heated on a water bath to 37-40°. The first fraction was precipitated by addition of 12-14 g of salt per 100 ml of serum solution; the second fraction was salted out from the centrifugate by bringing the concentration of the salt to 14-16 g per 100 ml. The third fraction was salted out at 16-18 g, the fourth at 18-20 g, and the fifth at 20-22 g per 100 ml of solution. The precipitate from each fraction was separated by centrifuging at a rate of 8000 rpm for 5-6 min; a completely clear centrifugate was not obtained. The precipitate was suspended in a phosphate-borate buffer, pH 6.8 which contained Na_2SO_4 in the above concentrations for each fraction and again separated by centrifuging. This operation was repeated three times,during which the amount of precipitate gradually decreased. Further, the reprecipitations were repeated three times for each fraction in the same concentrations of precipitating agent, but at different pH values of the phosphate-borate buffer (first fraction, pH 7.6; second fraction, pH 5.8; third and fourth fractions, pH 6.4, and fifth fraction, pH 7.8). The yield of the first fraction was 0.15 g of protein (dry weight); of the second, 1.0 g; of the third, 0.6 g; of the fourth, 0.2 g; and of the fifth, 0.1 g. Since the serum of different animals can have individual differences, it is sometimes necessary to carry out the reprecipitations at several different pH values to obtain electrophoretically homogeneous fractions.

The purified fractions were dissolved in the minimum volume of distilled water, dialyzed for 16-18 hours, first against distilled water,then against the buffer in which electrophoresis was run. Paper electrophoresis was carried out in 0.1 M boro-borate or veronal-medinal buffer at pH 8.6, voltage 130-150 v, and exposure 16-18 hours.

Instead of reprecipitation at determined pH values we can use treatment with aluminum hydroxide as follows: a solution of the fraction which contains 1.5-2.0% protein was mixed with an equal volume of 2% $Al(OH)_3$ by weight in a medinal buffer [32] and shaken for 30 min at a temperature not above 25°. Then the adsorbent was removed by centrifuging and the fraction was separated from the centrifugate by the corresponding Na_2SO_4 concentration.

The electrophoretic homogeneity of the first, fourth and fifth fractions was attained if treatment by $Al(OH)_3$ was run at pH 8.18; for the third fraction, at pH 8.68.

RESULTS

In Fig. 1 we give the electrophoretic control fractions obtained by the salt method described with reprecipitation both without maintenance of a required pH value and under conditions of controlled pH. In the first case (Fig. 1) we still have an example of a mixed fraction, while in the second case the electrophoretic homogeneity is indisputable. Such a result becomes possible if the centrifuging of the precipitated fraction is carried out at the rate given above. Isolation of the precipitate by filtration under these conditions is entirely unsuitable, since the electrophoretic control constantly shows the presence of 3-4 components.

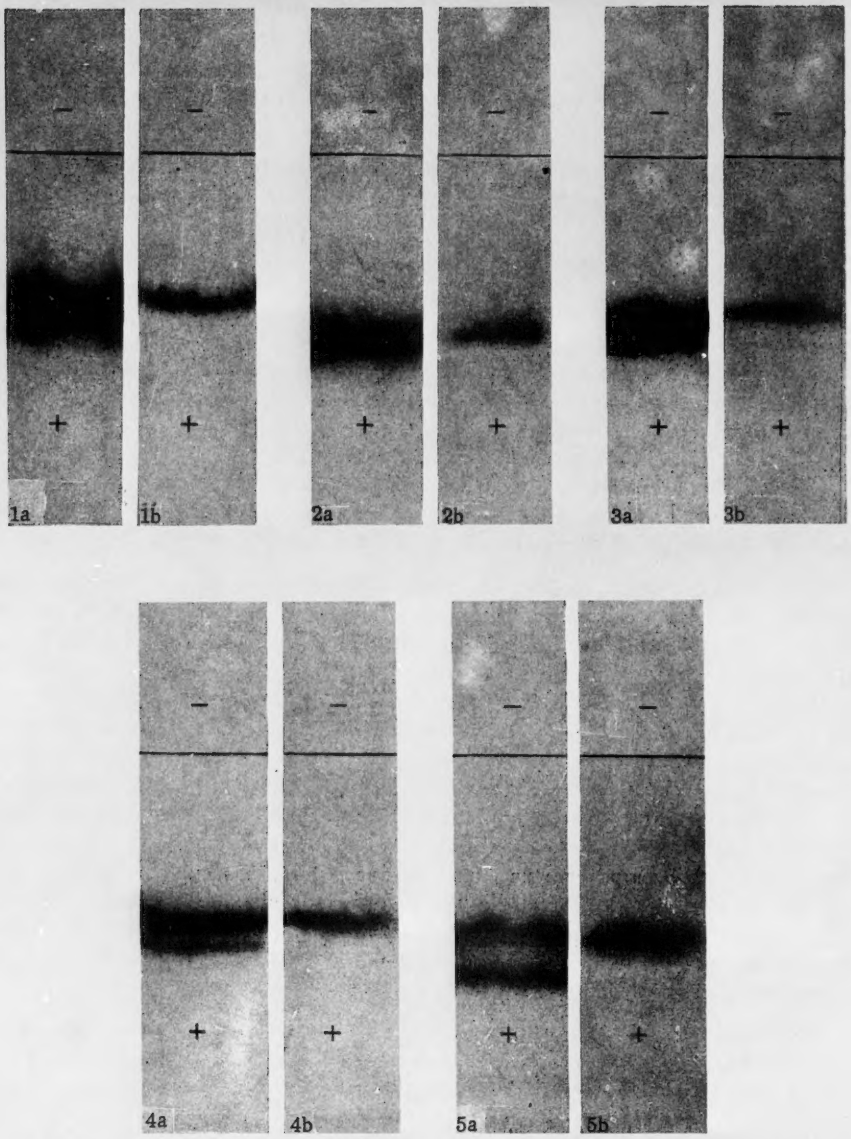

Fig. 1. Paper electrophoresis after salt precipitation of protein fractions of antidiphtheria serum. 1-5a) reprecipitation without maintaining a required pH value; 1 and 2a) upper zone γ_2-globulin, lower zone γ_1-globulin. 1-5b) reprecipitation under conditions of controlled pH; 3-5a) upper zone γ_1-globulin, lower zone β-globulin. 1b) γ_2-globulin; 2-5b) γ_1-globulin.

In Fig. 2 we give the electrophoretograms of antidiphtheria horse serum and the isolated protein fractions. The antidiphtheria serum was separated electrophoretically into albumin, α-, β-, γ_1-, and γ_2-globulins. According to electrophoretic mobility the first fraction is γ_2-globulin, the second to the fifth fractions belong to the γ_1-globulins.

As a result of our technique of preparative separation of the proteins of antidiphtheria serum there are obtained not only electrophoretically homogeneous fractions, but there is also assurance of their individuality, which is confirmed by later immunochemical and chromatographic studies.

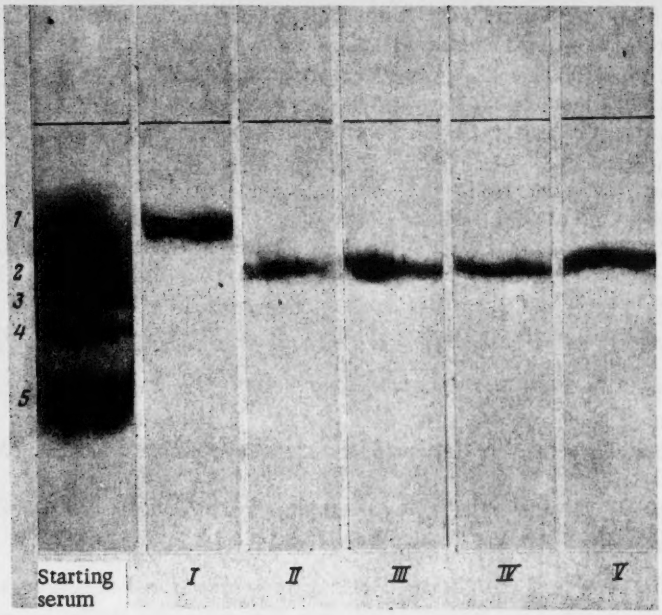

Fig. 2. Paper electrophoresis of antidiphtheria serum and purified fractions of γ-globulin. 1-5) zones of starting serum corresponding to γ_2-, γ_1-, β-, α-globulin and albumin. I-V) Similar salt fractions. I) γ-globulin, II-V) γ_1-globulin.

SUMMARY

We have worked out a method of successive separations of electrophoretically homogeneous γ-globulins of antidiphtheria serum by stepwise increases in concentration of sodium sulfate under conditions of strict control of pH in precipitation and reprecipitation of the protein fractions. We have obtained electrophoretically homogeneous γ_2-globulin and four fractions of γ_1-globulin from antidiphtheria horse serum.

The method of salt partition of the proteins of antidiphtheria serum into electrophoretically homogeneous fractions, with account of the time factor, is distinguished favorably from other methods by its simplicity.

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INDENTIFICATION OF NUCLEIC ACID COMPONENTS BY THE COMBINED METHODS OF ELECTRODIALYSIS AND CIRCULAR PAPER CHROMATOGRAPHY

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In the study of the carbohydrate components of nucleic acid hydrolyzates by the method of distributive paper chromatography it was noted that very often there were formed during hydrolysis substances which interfered with clear development of the carbohydrates, and the purine and pyrimidine bases were often superimposed on each other in a number of the solvents used.

We have worked out a method which permits the separation of the nucleic acid hydrolyzate into fractions of purine bases, pyrimidine bases, and carbohydrates, and then identification of the substances of each fraction. The method permits work with amounts of nucleic acid of 2.5 mg or more. It can also be used for preparative purposes.

EXPERIMENTAL

Hydrolysis of Nucleic Acids to Purine Bases and Pyrimidine Nucleotides

As the nucleic acid we used a commercial domestic RNA preparation, series 154, and an imported DNA preparation. A 10 mg sample was placed in an ampule and hydrolyzed with 0.5 ml of 1 N H_2SO_4 on a boiling water bath for one hour. To remove the H_2SO_4 we treated the hydrolyzate with a solution of $Ba(OH)_2$ to a neutral reaction to litmus. The $BaSO_4$ precipitate was separated by centrifuging and washed with hot water. The main solution and wash water were brought to a volume of 10 ml and submitted to electrodialysis.

Fractionation of the Hydrolyzate by Electrodialysis

In this work we used an electrodialyzer made of Plexiglas plates 10 mm thick. The apparatus consisted of two outer plates with dimensions 9 x 8 cm to which U-shaped plates were fastened (Fig. 1, A). Also there was one U-shaped plate which was inserted between the two outer ones.

The contiguous plate surfaces should be well polished, or else it is necessary to use a rubber washer, which is very undesirable. Each section has on the lower side an opening in which a rubber tube is inserted. This is joined to a glass tube fastened in a vertical position and serving to drain the liquid from the section. In all the parts at each angle there is an opening for a screw. In assembling the electrodialyzer, the sections are separated by a cellophane membrane and then the block is put together with screws and wing nuts.

In one of the outer sections of the electrodialyzer is placed a graphite electrode (anode) Fig. 1, B; in the other a platinum electrode (cathode). The middle section if filled with the hydrolyzate to be studied, the outer sections with distilled water. A constant current is used in running the electrodialysis. The initial voltage is 100 v, current strength 20 ma. In each running of an electrodialysis, the nucleic acid hydrolyzate was dialyzed for 25-30 min. At the end of this time, only carbohydrates remained in the middle section; the purine bases had migrated to the cathode section, the pyrimidines to the anode.

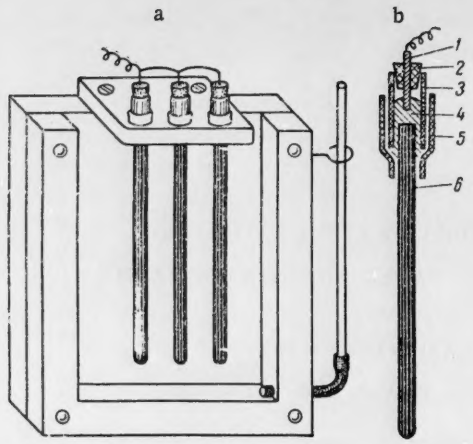

Fig. 1. Electrodialyzer. A) Anode section of electrodialyzer; B) plan for the carbon electrode. 1) Metal rod; 2) rubber stopper; 3) glass tube; 4) mercury; 5) rubber tube; 6) graphite rod.

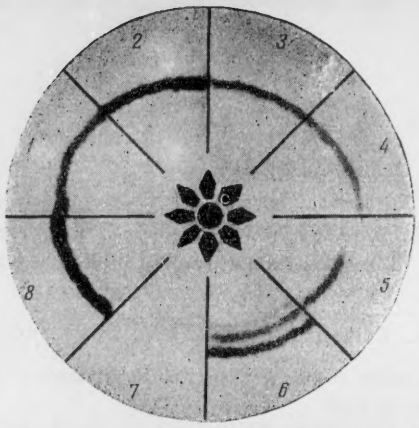

Fig. 2. Chromatogram of RNA hydrolyzate after 1 hour hydrolysis by sulfuric acid. 1, 3) ribose; 5) arabinose; 7) glucose; 6) mixture of ribose arabinose, glucose; 4,2,8) RNA hydrolyzates, concentration of carbohydrates in μg respectively equal to 16, 40, 80.

A sample of 2.5 mg of nucleic acid was submitted to an analogous treatment.

Study of the Fractions

Cathode fraction. The solution drawn from the cathode chamber was evaporated on a water bath. The dry residue was dissolved in 0.2 ml of distilled water (in the analysis of 10 mg of nucleic acid) and the solution was used for chromatographic study of the purine bases. Separation was carried out on a circular chromatogram. The circular chromatogram was a disk of Leningrad No. 2 paper, diameter 18 cm, divided into eight segments. At a distance of 1 cm from the center of the circle in each segment we deposited a solution of the substance to be studied in amounts of 0.01 ml, 0.005 ml, 0.002 ml, and 0.001 ml. Calculated on the nucleic acid this was, respectively, 500, 250, 100, and 50 μg of DNA and 400, 200, 80, and 40 μg of RNA (phosphorus content of RNA 7.95%). As a control we used a mixture of bases (guanine, adenine, thymine) and a solution of uracil.

The disk was placed in a desiccator on the bottom of which was poured an ammonia solution (to saturate the atmosphere). In a Petri dish we placed a mixture of n-butanol and 0.1 N solution of NH_3, 7:1. In an opening made in the center of the disk we inserted a wick (a small strip of chromatographic paper, tightly twisted), and we dipped one end of this into the vessel with the solvent. Separation continued for three hours. The purine bases were developed as dark spots in the ultrachemiscope of Brumberg, UB-1 [2].

The resulting chromatogram was photographed in ultraviolet light by a contact process recommended by Venkstern and Baev [3] but the exposure in our case was increased to 20 sec. Identification of the bases was carried out spectrophotometrically also. For this purpose the part of the chromatogram which corresponded to the separated spots of base was cut out, ground, and extracted with 5 ml of 0.1 N HCl for 24 hours at room temperature. As a control we used the eluate of the corresponding part of the control chromatogram. The absorption maximum in ultraviolet light was measured in a quartz cuvette 1 cm thick on an SF-4 spectrophotometer.

In all the samples studied we found guanine and adenine on the chromatograms. However, when we deposited a large amount of the nucleic acid solution being studied (0.01 ml, 0.02 ml) we detected not only purine, but also pyrimidine bases; cytosine in the analysis of RNA and cytosine and thymine in the study of DNA which had probably partly split from the pyrimidine nucleotide even by hydrolysis with 1 N HCl and H_2SO_4 for one hour. Uracil is probably also partly split out from the pyrimidine nucleotides of RNA, but in the system of solvents which we used it could not be separated from adenine.

Neutral fraction. The liquid drawn from the middle chamber was evaporated dry on the water bath. The dry residue was dissolved in 0.4 ml of distilled water. The solution was used for study of the carbohydrates by the chromatographic method. Separation was carried out also on a circular chromatogram which, in distinction from that used in study of the purine bases had on each segment a bridge 3-4 mm wide and 5 mm long; in addition the segments were separated from each other by 2 mm slit (Fig. 2). At the end of the bridge, joining it with the segment, were placed different amounts of the solutions studied: 0.001, 0.002, 0.005, 0.01, and 0.02 ml. As a control we used solutions of ribose, arabinose, glucose, and their mixture.

Separation was carried out in a mixture of n-butanol-CH_3COOH-H_2O (4:1:5) for three hours. The spots of the sugars were developed with ammoniacal silver solution. We could easily see ribose on the chromatogram even when we deposited 0.001 ml of RNA solution which calculated on the ribose corresponded to about 8 μg of the sugar.

It follows from the above that separation of the carbohydrates on a circular chromatogram is a very suitable method which requires very little time and has a high sensitivity.

However, as Orlova mentioned, when this method is used, it is necessary to consider the following fact: when the solvent passes along the paper disk, it is not distributed in the same way in all directions. The greatest rate of movement of the solvent follows the mechanical direction of the paper fibers. Thus the solvent is not distributed on the paper in the form of a circle, but of an ellipse (Fig. 2), the length of whose axis agrees with the course of the fibers. Therefore when we deposit the solution being studied and the standard soltuion of sugars, it is necessary to arrange them in a definite order. For comparison, they must be deposited in pairs on the following segments: 1 and 8, 2 and 3, 4 and 5, 6 and 7 (Fig. 2). It is necessary to compare the results by depositing the standard and studied solutions on segments 1 and 2, 5 and 6, etc.

Anode fraction. The solution from the anode chamber, which contained pyrimidine nucleotides, was evaporated to dryness. The dry residue (10 mg) was again hydrolyzed with 0.5 ml of 70% perchloric acid and transferred to a tube with a ground glass stopper. The solution was hydrolyzed for one hour at 100°. The hydrolyzate was diluted with twice the volume of water and treated in the cold at first with 20% and then with 2% KOH to remove the $HClO_4$. The precipitate of $KClO_4$ was separated by centrifugation. The solution after centrifuging was brought to a volume of 10 ml and submitted to electrodialysis.

For the study of the pyrimidine bases we used the cathode fraction. The solution was evaporated dry, the dry residue was dissolved in 0.2 ml of distilled water and studied chromatographically. Chromatographic analysis, detecting and identifying the pyrimidine bases, was carried out in the same way as was described for the purine bases. We detected on the chromatogram cytosine and uracil in RNA and cytosine and thymine in DNA.

In the investigation of 2.5 mg of nucleic acid, hydrolysis and later treatment of the fractions were dissolved in 0.1 ml of distilled water and we deposited on the chromatogram 0.01, 0.02, and 0.03 ml of solution. The results corresponded to the results obtained in the study of larger quantities.

SUMMARY

We have worked out a method which permits separation of nucleic acid hydrolyzates into fractions of purine and pyrimidine bases and a carbohydrate fraction by the use of electrodialysis.

The fractions studied, obtained by the use of electrodialysis, were treated by the method of partition chromatography on a circular chromatogram. Separation on the circular chromatogram occurred more quickly than usual and the method was more sensitive.

Combination of the methods of electrodialysis and circular chromatography permits work with small amounts of nucleic acids.

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HEMATIN CATALYSIS OF THE OXIDATION OF VITAMIN A AND FATS

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The oxidation of vitamin A at the double bonds is markedly hastened by peroxides and fatty acid radicals, the formation of which in fatty tissues is catalyzed by different heme compounds [1-3]. However, the conditions of such catalysis have been little studied. It is assumed [3-5] that hemes show their catalysis function in the form of oxidized derivatives, hematins which contain trivalent iron, compounds of the ion bound to hydroxyl. Such hematins form catalytically active complexes with hydrogen peroxide or alkyl peroxides. In catalytic oxidation of lipids by hemoglobin, catalytic activity is shown by the methemoglobin formed from it.

Developing these ideas in relation to the internal organs of animals in which, due to oxidative enzymatic processes, there is formation of H_2O_2 and accumulation of methemoglobin, we have studied the conditions for an active hematin catalyst and the possibility of preventing the formation of a catalytically active hematin in order to stabilize fats and vitamin A. The investigation of heme catalyzed oxidation of lipids has not yet been carried out in this direction.

Oxidation of vitamin A in the liver and other internal organs of fish has often been observed, but the catalytic effect has been wrongly attributed to lipoxidase [6]. The destruction of carotene by an extract of rat intestine at pH 3 has also been ascribed to lipoxidase [7], although it is inactive at such a pH value. Recently the absence of lipoxidase in animal tissues has been established [8]. In the oxidation of vitamin A at the double bond epoxides are formed [9,10] and polyenes of different structures [11,12]. The oxidation of vitamin A in the organism is practically irreversible, as indicated by the lack of biological activity of the epoxides [13].

The purpose of the present work was to determine the possibility of oxidation of vitamin A in fatty tissues with participation of hemocatalysts.

METHOD OF STUDY

Oxyhemoglobin and methemoglobin were obtained from fresh rabbit blood [14], cytochrome c from beef cardiac muscle [15], aldehyde oxidase from swine liver [16], vitamin A aldehyde from crystalline vitamin A acetate [16].

To a phosphate-citrate buffer were added varying amounts of oxidation catalyst or homogenates of animal organs and 1 ml of toluene which contained 100 mg of cod liver oil and 3 mg of vitamin A (10,000 I.U.). After placing in a thermostat, vitamin A was determined by reaction with antimony trichloride or by spectrophotometry with corrected extinction [17]. In the experiments on rats methemoglobin was determined in blood by the method of Gorn [18]. Blood was obtained from the caudal vein.

RESULTS

Comparison of the catalytic activity of the different heme compounds and metals was made by determination of the time required for destruction of half the vitamin A. The hematin compounds had the greatest activity.

Vitamin A was oxidized almost twice as fast by neutral methemoglobin as by oxyhemoglobin; for the same catalytic activity it was necessary to increase the concentration of the latter five fold compared to the methemoglobin concentration (Table 1). A still higher catlytic activity was shown by "acid hematin" which is formed from oxyhemoglobin in a buffer solution, pH 4.0.

TABLE 1

Comparative Activity of Different Catalysts for Oxidation of Vitamin A. Incubation at pH 5.7 and 20°

Catalyst	Mole concentration	Time of destruction of half the vitamin, hours
Acid hematin, pH 4	$4 \cdot 10^{-5}$	15
Methemoglobin	$4 \cdot 10^{-5}$	21
Oxyhemoglobin	$4 \cdot 10^{-5}$	40
Oxyhemoglobin	$2 \cdot 10^{-4}$	22
Cytochrome c	$4 \cdot 10^{-5}$	60
Fe^{++}	$1 \cdot 10^{-1}$	60
Fe^{+++}	$1 \cdot 10^{-1}$	75
Cu^{++}	$1 \cdot 10^{-1}$	55
Co^{++}	$1 \cdot 10^{-3}$	65
Control, buffer solution	–	>240

The catalytic activity of cytochrome c under these conditions is one and a half times less than for oxyhemoglobin. The catalytic activity of the ions of heavy metals is marked only at concentrations more than 100 times greater than the living tissues.

In connection with these results on the high catalytic activity of acid hematin, it is of interest to study the rate of oxidation of vitamin A by solutions of oxyhemoglobin depending on pH. The results of the experiments are given in Fig. 1.

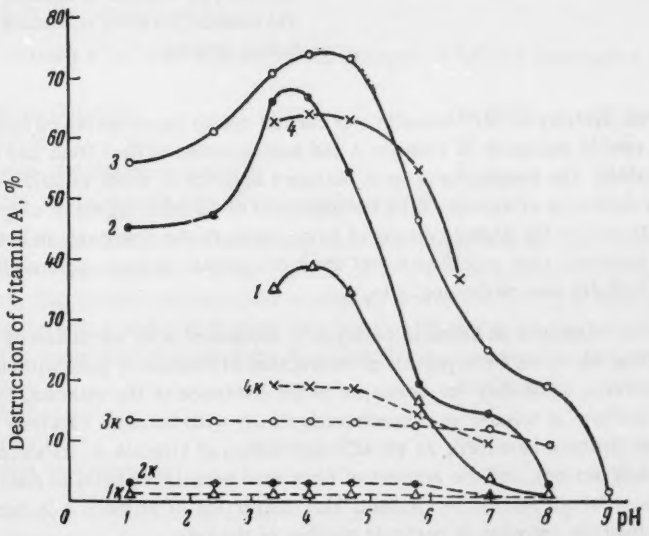

Fig. 1. Kinetics of oxidation of vitamin A in the presence of oxyhemoglobin depending on pH. Concentration of oxyhemoglobin $4 \cdot 10^{-5}$ M. 1) Incubation at 37°, 7 hours; 2) 14 hours, 3) 62 hours, 1k, 2k, 3k, corresponding controls with buffer solutions; 4) incubation at 60°, 3.5 hours; 4k) control with buffer solutions.

The increased catalytic activity of oxyhemoglobin when the solution was acidified to pH 4.0 was related to increased rate of formation of acid hematin in the catalytic process. Acidification of the oxyhemoglobin solution to pH 4.0 caused a change in the visible absorption spectrum in the region characteristic for neutral methemoglobin and hastened the denaturation of globin in the process of catalysis with formation of acid hematin (parahematin globin or denatured ferrihemochromogen globin). With further acidification of the solution there occurred rapid formation of acid hematin and its destruction in the process of catalysis, so that the rate of oxidation of vitamin A decreased. The greatest destruction of vitamin A occurred in the first seven hours and after 14 hours the rate of oxidation fell sharply in connection with the destruction of acid hematin. At 60° the rate of oxidation of vitamin A was about 10 times higher than at 37°.

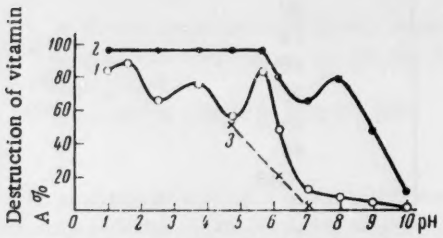

Fig. 2. Kinetics of oxidation of vitamin A in the presence of beef liver homogenate and of the internal organs of carp depending on pH. 1) Homogenate of beef liver at 37°, 39 hours; 2) the same, 66 hours; 3) homogenate of internal organs of carp at 20°, 24 hours.

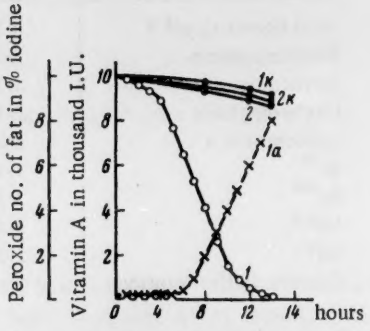

Fig. 3. Relation between rate of oxidation of vitamin A and accumulation of fatty acid peroxides. Concentration of acid hematin 7.10^{-5} M; pH 4.0 at 32°. 1) Vitamin A in cod liver oil in toluene; 1a) peroxide number of fats; 1k) control, vitamin A in oil with buffer solution; 2) vitamin A acetate in toluene 2k) control, vitamin A acetate with buffer solution.

A study of the catalytic activity of acid hematin was carried out by experiments on homogenates. Fig. 2 shows the relation between rate of oxidation of vitamin A and homogenates of beef liver and of the internal organs of carp and the pH values. The homogenates were obtained at ratios of tissue to buffer solutions 1:4. The considerably greater rate of oxidation of vitamin A by homogenates of internal organs of carp than by homogenates of beef liver is explained by the higher content of hemoglobin in the first case. In both cases destruction of the vitamin was sharply increased with acidification of the homogenate, beginning from pH 5.7 in connection with the formation of acid hematin and methemoglobin.

The rapid destruction of vitamin A in hematin catalysis is connected with formation of active peroxides and radicals of fatty acids (Fig. 3). At first the process of destruction of vitamin A goes without accumulation of a quantity of fatty acid peroxides, since they are destroyed in the oxidation of the vitamin. In the absence of fats oxidation of vitamin A acetate in toluene goes much more slowly with hematin catalysis and differs little from the control experiments (without hematin). At 40-45% destruction of vitamin A, its antioxidant power is neutralized by the prooxidizing actions, and the amount of fatty acid peroxides begins to rise; with their further increase there is a proportional fall in amount of vitamin. The destruction of vitamin A in hematin catalysis is thus a more accurate index than the increase of peroxide number of the fats.

The rate of oxidation of vitamin A depends on the concentration of acid hematin (Fig. 4). The showing of a linear relation between rate of vitamin A oxidation and concentration of hematin catalyst to the power 0,5 is a characteristic index of a chain process of oxidation.

The high catalytic activity of acid hematin is of great significance for keeping crude fats, but in the organism acid hematin is normally absent and appears only in disease and poisoning connected with hemolysis

(malaria, poisoning by acids, etc.). There is more general significance in neutral methemoglobin, which is usually present in small amounts in the blood. We determined the catalytic activity of methemoglobin depending on the pH of the medium when it was formed from oxyhemoglobin under the influence of potassium ferricyanide ($1 \cdot 10^{-2}$ M) [19]. The results of the experiments are given in Fig. 5. The catalytic activity of neutral or acid methemoglobin is a maximum at pH 5.5. Further acidification of the methemoglobin solution leads to a decreased rate of destruction of the vitamin because of the more rapid destruction of the methemglobin during the catalysis. Alkaline methemoglobin is only slightly active catalytically.

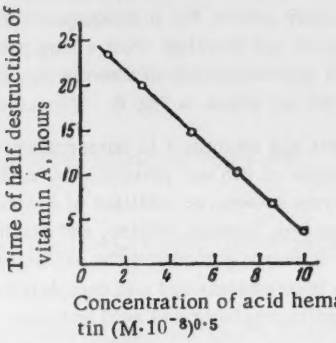

Fig. 4. Relation of rate of oxidation of vitamin A in oil to concentration of acid hematin. Conditions of oxidation: pH 4.0 at 32°.

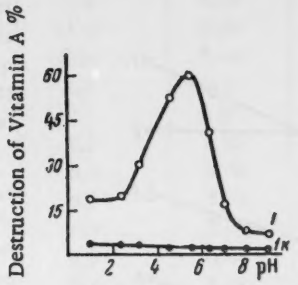

Fig. 5. Oxidation of vitamin A in the presence of methemoglobin depending on pH. Conditions of oxidation: 14 hours at 37°. 1) oxyhemoglobin $4 \cdot 10^{-5}$ M plus potassium ferricyanide $2 \cdot 10^{-2}$ M; 2) control, potassium ferricyanide $2 \cdot 10^{-2}$ M in buffer solution.

For an approximation to biological conditions we determined the catalytic activity of methemoglobin formed at pH 6.8 from oxyhemoglobin with participation of the system aldehyde oxidase-acetaldehyde. The H_2O_2 formed in oxidation of acetaldehyde caused formation of methemoglobin which hastened the catalytic oxidation of vitamin A in the presence of fats.

The content of vitamin A (in % of original value) changed as follows (incubation at pH 6.8, 20°)

Oxidation Catalyst	24 hours	48 hours
Oxyhemoglobin $4 \cdot 10^{-5}$ M	98.2	93.0
The same plus acetaldehyde (control)	95.1	79.4
The same plus acetaldehyde plus acetaldehyde oxidase	79.1	45.6

Further study of the catalytic role of methemoglobin was carried out on beef liver homogenates by determination of the destruction of the vitamin A in the liver at 20°. The homogenate was prepared without addition of water or buffer solution. The initial concentration of vitamin A was 410 I.U./g of homogenate. For the formation of methemoglobin we added to 8 g of homogenate 1 ml of 2% $NaNO_2$ or 1 ml of 2% acetaldehyde (incubation at pH 6.9, 20°). The content of vitamin A (in % of initial value) changed as follows:

Addition	24 hours	48 hours
Beef liver homogenate	94.1	80.2
The same plus $NaNO_2$	49.3	16.6
The same plus acetaldehyde	56.0	46.2

These results show that under the influence of methemoglobin, vitamin A in the beef liver homogenate is quickly destroyed. Methemoglobin in animal organs in a number of cases can form very rapidly and cause destruction of vitamin A and oxidation of fats. For example, in homogenates of the internal organs of such fish as carp, pike perch, and bream, 70-80% of the vitamin A is destroyed in 24 hours at 20°, but when the blood is removed (by washing the organs) the destruction of vitamin A in the same time is reduced by 5-20%.

It is interesting to observe the role of fats in the oxidation of vitamin A in the homogenates. The addition of 10-20% of sunflower oil to the beef liver homogenate hastened the oxidation of vitamin A several fold; destruc-

tion of vitamin A in chicken liver homogenate after 24 hours at 20° was about 10%, but in the case of a high fat content in the liver (20-30%), destruction of vitamin A rose to 40%. All this can easily be understood in connection with the role of fatty acids in the transfer of peroxide oxygen to vitamin A.

In characterizing the peculiarities of catalysis by neutral methemoglobin and acid hematin it is most necessary to emphasize that in both cases iron of the heme is oxidized to the trivalent, catalytically active state. Actually, determination of the spectral properties of both catalysts showed their close similarity. The difference between the catalysts consisted in the degree of denaturation of the globin protein, and the difference in catalytic properties is that the acid hematin is more active, but is decomposed more quickly than methemoglobin and therefore after a long period of time the latter oxidizes a greater amount of vitamin A. The results of these experiments are shown in Fig. 6.

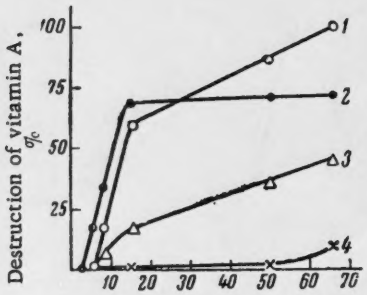

Fig. 6. Kinetics of oxidation of vitamin A in the presence of different heme proteins. Concentration of heme protein $4 \cdot 10^{-5}$ M at 37°. 1) Methemoglobin, pH 5.7; 2) acid hematin, pH 4.0; 3) oxyhemoglobin, pH 5.7; 4) control buffer solution, pH 5.7.

The oxidation of fats and vitamin A in homogenates of beef liver and internal organs of fish was prevented by addition of 0.4 M solutions of enzyme poisons: on addition of cyanide, practically completely, and for fluoride, sulfide, and bisulfite, partially. Carbon monoxide scarcely hindered the oxidation of vitamin A and fats in the homogenates and was completely ineffective in the case of methemoglobin and acid hematin.

From the above results of the high catalytic activity of hematins at pH 4.0 and 5.5 and the decreased activity at pH 7 it follows that the simplest process for stabilizing lipids is to bring the pH of the animal tissues to neutral or weak alkalinity with $NaHCO_3$ solution. However, this does not completely prevent catalytic activity of the already formed or forming methemoglobin. For reduction of the iron of methemoglobin to the divalent state with formation of the catalytically slightly active heme compounds, we used ascorbic acid, neutralized before the experiment with $NaHCO_3$ to a pH of 6.5-7.2, or with sodium hyposulfite. These reducing agents were added in the amount of 0.05-0.2% in the form of 5-10% aqueous solutions. The addition of these reducing agents was desirable when it was necessary to heat the tissue for inactivation of the enzymes [20], since at higher temperatures the catalytic activity of the hematins is sharply increased. It is also effective to add 0.1% gallic acid before heating. The results of these experiments are given in Table 2.

After treatment, the homogenates were kept at 20°. The initial content of vitamin A in the internal organs of the bass was 740 and in the case of the cod livers, 250 I.U./g of homogenate.

Table 2 shows that the use of formaldehyde as an antiseptic leads to rapid destruction of vitamin A, evidently caused by formation of methemoglobin. Heating the raw material stops oxidation of vitamin A in the same way as a shift in pH to the alkaline side with soda. It is most effective before heating to add ascorbic acid, sodium ascorbate, hyposulfite and gallic acid in the amount of 0.2%. In heat treatment of the raw material, besides the inhibition of hematin catalysis, there is inactivation of lipase, which stops the increase of acid number of the fat.

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We also tried the effectiveness of heat treatment in production experiments at Murmansk. In the usual method of preserving the organs of bass by salting with 10-20% NaCl and then keeping for 10 days at 5-10°, 20-25% of the vitamin A is destroyed and the acid number of the fats rises to 15. With heat treatment of the same organs at 90° for 15 min with addition of 5% salt as the preservative and under the same conditions of keeping almost no destruction of vitamin A is found and the acid number of the fats is 2.3. The addition of 0.05% ascorbic acid to the emulsified mass of cod livers in preparing a special form of canned food [21] increases the retention of vitamin A in sterilization by 10-15%. It should be noted that an increased amount of added ascorbic acid in the liver over that mentioned causes formation of a green bile pigment and produced an undesirable worsening in the outward appearance of the food. The formation of the pigment occurs as a result of combined oxidation of hemoglobin and ascorbic acid [5,22].

TABLE 2

Effect of Conditions of Treatment of Internal Organs of Bass and Cod Livers on Content of Fats and Vitamin A

Conditions of treatment	Time of keeping days	Acid no. in mg KOH/g fat	Peroxide no. in % iodine	Vitamin A in % initial content
Internal organs of bass salted (10% NaCl)	1	5.8	0.04	100
	6	11.0	0.99	55.1
	13	20.3	1.04	40.0
The same + formaldehyde (0.3%; in 3% solution)	1	5.8	0.10	100
	6	6.1	1.80	43.3
	13	8.5	2.25	20.5
The same + heating at 90°, 5 min	1	5.8	0.08	100
	6	5.8	0.50	88.2
	13	5.8	0.55	77.2
The same + Na_2CO_3 (2%, in 20% solution)	1	5.8	0.03	100
	6	2.4	0.18	95.1
	13	13.6	0.47	85.2
The same + hyposulfite (0.2%, in 10% solution) + heating at 90°, 5 min	1	5.8	0.07	100
	6	5.8	0.17	96.3
	13	5.8	0.44	89.1
The same + gallic acid (0.1%) + heating at 90°, 5 min	1	5.8	0.05	100
	6	5.8	0.05	97.2
	13	5.8	0.45	89.3
Cod liver salted (10% NaCl)	6	5.9	0.02	80.1
The same + formaldehyde (0.3%, in 3% solution)	6	2.4	0.07	69.2
The same + heating to 90°, 5 min	6	0.8	0.04	90.0
The same + ascorbic acid 0.1% + heating at 90°, 5 min	6	0.9	0.01	97.0
The same + Na ascorbate 0.15% + heating at 90°, 5 min	6	0.9	0.01	98.1

Methemoglobin catalysis can also be of significance in the oxidation of vitamin A in the animal organism. A study of this question was carried out on white rats maintained on the usual laboratory rations. For three days the rats were given a peroral dose of vitamin A as an emulsion and two hours after feeding were injected subcutaneously with 60 mg/kg weight of animal of 2% $NaNO_2$. After two hours methemoglobin was determined in the blood from the caudal vein. At this time the content of methemoglobin and vitamin A in the blood were maximal. On the fourth day the animals were killed and the vitamin A was determined in the liver and blood plasma spectrophotometrically.

In one of the control groups the animals were fed vitamin A without injecting $NaNO_2$; in another for three days only $NaNO_2$ was injected and on the next three days vitamin A was fed. The purpose of the second control experiment was to show whether there was a decrease in assimilation of vitamin A because of weakening the animals by injection of $NaNO_2$. The results are given in Table 3.

The table shows that the accumulation of vitamin A in the liver of rats is lower, the greater the methemoglobin content of the blood. Spectrophotometric determination of vitamin A in blood showed that with increasing methemoglobin in the blood the quantity of oxidized products of the vitamin was raised; that is, the ratio of the extinctions E_{310}/E_{325} increased. On the other hand, in the control experiments the accumulation of vitamin A in the liver was uniformly high. Therefore we can conclude that the formation of methemoglobin in the organism hastens the destruction of vitamin A.

DISCUSSION

These experiments have shown that the catalytic activity of oxyhemoglobinand methemoglobin with respect to oxidation of fat and vitamin A is increased at acidifications of the solutions corresponding to 4.0 and 5.5. This can be explained by the fact that acidification of the solutions causes oxidation of the heme iron to the trivalent state, dissociation of hemoglobin, and decrease in the energy of denaturation of globin [22].

The practical use of this case of stabilizing fats and vitamin A by preventing the formation of catalytically active hemoproteins is of value not only for raising the quality of the raw materials produced as sources of vitamin A, but also for other forms of fat sources. The treatment of beef fat tissues with 1% $NaHCO_3$ will improve the quality of the melted down fat while the use of 0.5% CH_3COOH will sharply worsen the quality of the fat [23]. A positive result is also obtained by treatment of different types of meat products with solutions of ascorbic acid and heat [24].

In our experiments on destruction of vitamin A in the organism by injection of $NaNO_2$ this is certainly related to formation of methemoglobin and specific peroxidase catalysis, though this does not exclude the possibility of other reactions. In the experiments of a number of authors [25] on feeding poultry and pigs on herring meal with a high content of $NaNO_2$ (about 0.1%) the formation of methemoglobin in the blood was also noted, with decrease in the storage of vitamin A in the animal livers. Rapid destruction of vitamin A in humans in a number of diseases [11] is probably also connected with formation of methemoglobin which again is increased by sulfonamides and other medicinal preparations.

TABLE 3

Effect of Methemoglobin on Destruction of Vitamin A in Experiments in vivo

Experimental conditions	Methemoglobin content in rat blood in % of hemoglobin	Accumulation of vitamin A in liver	
		in I. U.	in % of dose given
Feeding vitamin A (control I)	1 – 2	16,600	51.9
The same + injection of $NaNO_2$	7 – 18	15,400	48.2
The same + injection of $NaNO_2$	30 – 40	12,450	39.0
Injection of $NaNO_2$ for 3 days, next 3 days feeding vitamin A (control II)	1 – 2	16,600	51.9

In the animal organism vitamin A is present not only in the form of an alcohol or ester, but also as an aldehyde. Vitamin A aldehyde is oxidized with participation of methemoglobin and fats at the double bonds, and in the presence of aldehyde oxidase at the aldehyde groups with formation of vitamin A acid [16]. The general scheme of destruction of vitamin A in the organism can be represented as follows:

carotene → vitamin A aldehyde → vitamin A alcohol

↓ ↘ ↓

vitamin A acid → epoxide and products of oxidation of vitamin A

SUMMARY

We have established the high catalytic activity of neutral methemoglobin and acid hematin in oxidation of vitamin A and fats in experiments in vitro and in vivo. The optimum pH of action of the catalysts is 5.5 and 4.0 respectively. We have studied the kinetics of catalytic oxidation of vitamin A and fats and suggested different processes for their stabilization on the basis of preventing formation of the catalytically active hemoproteins.

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THE CHANGE IN CONTENT OF SULFHYDRYL GROUPS IN PROTEINS OF THE STRUCTURAL ELEMENTS OF RAT LIVER CELLS IRRADIATED WITH ROENTGEN RAYS

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The results of our previous work showed that in the early period after irradiation there was a decrease in the quantity of SH groups in the soluble proteins of the liver and spleen of irradiated rats [1,3]. A decreased quantity of SH groups in liver proteins of irradiated rats was also found by Yakovlev and Isupova [7]. Bankowsky [8] by a histochemical method found a decreased content of SH groups in liver proteins and epidermis of irradiated mice. Ono [11] observed a decreased content of SH groups in preparations of isolated mitochondria and chromosomes after their irradiation in vitro. In the fractionation of liver proteins of irradiated rats it was found that the decreased content of SH groups in different electrophoretic fractions of these proteins was not the same [4] and was chiefly connected with nonsecreted liver proteins.

In this connection it seemed to be of interest to study changes in the content of SH groups in proteins of the different structural elements of liver cells.

METHODS

The experiments were carried out on male white rats weighing 180-200 g. The irradiation was produced in an RUM-3 apparatus. The dose of irradiation in vivo and in vitro experiments was 1500 r at a dose strength of 50 r/min, 195 kv, 15 ma. Filters 0.5 mm Cu and 1 mm Al. Skin focus distance 30 cm. The rats were killed by decapitation after 10, 30, and 60 min, and on the first, second and third day after irradiation. The livers were removed and perfused first with cooled physiological saline, and then with a 0.25 M solution of sucrose. Separation of the subcellular structures was carried out by the method of Hageboom and Schneider with the modification of Khesin [9,5]. Differential centrifuging was carried out in a refrigerated centrifuge. Nuclei were precipitated at 150 g, mitochondria at 16,000 g, and microsomes at 21,000 g.

It is not impossible that in the microsome fraction there precipitated also the so-called light large granules [6]. The isolated structures were washed with 0.25 M solution of sucrose, homogenized in distilled water, and centrifuged. In the supernatant liquid we determined the protein concentration by the method of Lowry [10] and the quantity of SH groups. Amperometric titration of the SH groups was carried out with 0.001 M $AgNO_3$ in an ammonia buffer.

Besides the experiments with irradiated animals we ran experiments in vitro in which we irradiated the structural elements of the cells isolated from livers of nonirradiated rats, and also the water solutions of proteins obtained from them. The structural elements were isolated from 9 g of liver (wet weight). After washing with 0.25

M sucrose solution the precipitate was transfered to a glass container in which irradiation was carried out. The protein solutions isolated from the structural elements of liver cells were brought to the same concentration (0.5 mg protein/ml) before irradiation.

RESULTS

The content of SH groups in water soluble proteins of nuclei and cytoplasmic elements of the cells isolated from liver homogenates of control and irradiated rats are given in the table.

Change in Content of SH Groups in Proteins of the Structural Elements of Rat Liver Cells at Different Periods after Irradiation

Soluble protein of cell structure	Control		Period after irradiation											
			10 min		30 min		60 min		1 day		2 days		3 days	
	I	II	I	II	I	II	I	II	I	II	I	II	I	II
Nuclei	0,61 (31)	100	0,53 (8)	87 <0,01	0,49 (6)	80 <0,01	0,47 (8)	77 <0,01	0,48 (8)	79 <0,01	0,45 (8)	74 <0,01	0,46 (8)	75 <0,01
Mitochondria	0,68 (30)	100	0,66 (8)	97	0,65 (6)	96	0,68 (8)	100	0,59 (8)	87 <0,01	0,62 (8)	91	0,57 (8)	84 <0,01
Microsomes	0,67 (30)	100	0,70 (8)	104	0,74 (6)	110	0,73 (8)	109	0,63 (8)	93	0,49 (8)	73 <0,01	0,49 (8)	73 <0,01
Centrifugate	0,74 (30)	100	0,79 (8)	107	0,65 (6)	88	0,63 (8)	85 <0,01	0,62 (8)	84 <0,01	0,65 (8)	88	0,68 (8)	92

Note I: Amount of SH groups in percent cysteine; figures in parentheses, number of animals in experiment. II: Upper number, quantity of SH groups in percent of control; lower number, significant differences.

In the study of livers of control unirradiated animals we found the SH group content shown in the centrifugate proteins to be lowest in proteins of the nuclei. After 10 min from the irradiation, the statistically significant ($p < 0.01$) decrease in content of SH groups was noted only in proteins of the nucleus. After 60 min the content of SH groups in the nuclear proteins fell by 23% compared to the controls and in longer periods did not change much further. After 60 min from the irradiation a decrease in quantity of SH groups was observed in proteins of the centrifugate (by 15%, $p < 0.01$) and after a day a slight decrease in amount of SH groups was seen in proteins of the mitrochondria. After two days the content of SH groups in proteins of the microsomes fell by 27%.

A study of the effect of irradiation on content of SH groups in proteins after irradiation of isolated structures in vitro showed the following:

Cell Structure	Control	Experimental
Nuclei	0.61	0.51
Mitchondria	0.68	0.63
Microsomes	0.69	0.75
Centrigugate	0.72	0.66

Thus, after irradiation a marked fall in amount of SH groups was found only in proteins of the nuclei (by 16%). In proteins of the protoplasmic elements a definite change was not observed.

In experiments with irradiation in vitro of protein solutions isolated from the corresponding structures we obtained the following results:

Proteins of cell structures	Before irradiation	After irradiation
Nuclei	0.61	0.46
Mitochondria	0.73	0.61
Microsomes	0.78	0.42
Centrifugate	0.76	0.68

Thus under these conditions we find a decreased amount of SH groups in proteins isolated from microsomes (by 47%) and somewhat less in nuclear proteins (by 25%).

DISCUSSION

The results of the work indicate that irradiation of the animals decreases the content of SH groups of some of the proteins isolated from their tissues. This agrees with the results of our earlier published studies [1,3,4]. In study of the water soluble fraction of the proteins of the structural elements of liver cells in short periods after irradiation of the animals, a decreased content of SH groups was noted only in proteins of the cell nuclei. In proteins of the other cellular elements this change was found in later periods. The change in SH groups in nuclear proteins occurred also in irradiation of isolated cell structures. When we irradiated solutions of proteins isolated from the studied structures, the proteins of the microsomes had SH groups most sensitive to irradiation.

Our results show that the reactivity of the SH groups of the proteins may depend not only on the characteristic structures of the protein molecules themselves, but also on the supermolecular structures in which are included the protein molecules of the tissue. On the basis of the changes in quantity of SH groups in the proteins of cell nuclei in short periods after irradiation we can conclude that the nuclei are the most radiosensitive structures of the cell. This agrees fully with the existing data on destruction of DNA synthesis in irradiated tissues and also with the antimitotic action of irradiation.

The explanation of how much this observed inactivation of SH groups of water soluble proteins of the nuclei is connected with interruption of DNA synthesis and inhibition of mitosis will be the purpose of our further study. However, it is interesting to see that there have been repeated mentions of the role of SH groups in the process of cell division [2].

SUMMARY

In a short period after irradiation of animals there is a decrease in content of sulfhydryl groups of the water soluble proteins of the cell nuclei in the liver by 20-25%. The decreased quantity of sulfhydryl groups in the water soluble proteins of the cytoplasmic elements is noted after a longer period of time.

When water solutions of proteins isolated from the structural elements of liver cells are irradiated in vitro, the most marked decrease in quantity of SH groups is found in the proteins of the microsomes (by 47%) and in nuclear proteins (by 25%).

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PARTICIPATION OF AMINO ACIDS IN THE REACTION OF MELANOIDINE FORMATION IN BREAD BAKING

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According to a hypothesis which has been advanced earlier [1], the darkly colored substances of bread crust are formed as a result of oxidation-reduction reactions of reducing sugars with amino acids and other amino compounds, leading to the formation of melanoidines. Auerman, Kretovich et al. [2] have obtained indirect evidence in support of the fact that the reaction of melanoidine formation during bread baking occurs, in the first place, at the expense of the products of hydrolytic breakdown of proteins. However, these authors did not carry out a direct determination of the degradation products of proteins of bread.

EXPERIMENTAL

The low amino acid content in bread, as well as the presence of considerable amounts of substances interfering with the estimation of amino acids rendered the experimental work difficult. It is necessary to note that extraction with 80% ethanol, which is usually carried out during the quantitative determination of free amino acids in plant material, leads, in the case of flour and dough, to the simultaneous solution of vegetable protein and sugars, which interferes with the isolation of amino acids.

The method we propose for the quantitative determination of free amino acids in rye bread and flour is based on extraction of free amino acids with water, while cooling with ice, precipitation of dissolved proteins, mucilage, and dextrins with ethanol, separation of amino acids from sugars and salts on a column of cation-exchanging resin KU-2, elution of the amino acids from the resin with alcoholic and aqueous ammonia, concentration of the solution of amino acids in vacuum, and determination of the amino acids by quantitative paper chromatography according to Kretovich and Uspenskaya [3,4].

Description of the procedure

Preparation of the KU-2 resin. KU-2 resin (standard grade, containing 8% of divinylbenzene bonds) was air-dried for 24 hr, and milled to pass through a 120-mesh sieve. It was necessary to avoid heating the resin too much during milling. Then, the resin was washed five times with distilled water in a glass cylinder, stirring with a glass rod, and allowing to settle each time for 15 min. The particles which did not settle in 15 min were discarded, and the residue was stirred again with a new portion of water, etc. The material was then transferred to a separating funnel, the bottom of which was plugged either with a thick layer of glass wool, or with a perforated disc covered with filtered paper, and washed with a 10% NaCl solution prepared with a distilled water that was boiled and cooled. This solution must be neutral to the mixed indicator (methyl red+ methylene blue) and litmus.

The 10% NaCl solution was passed through the resin for several days, until the eluate became neutral (bottle color with the mixed indicator). Then, the resin was washed with boiled distilled water until the eluent was free of Cl^-, as tested with 6% $AgNO_3$. The resin was further washed with 2N HCl, prepared also with boiled distilled water, until the eluate was acid to the mixed indicator. Finally, the resin was washed with boiled distilled water until the eluate was neutral. This process was repeated thrice. As a result of this treatment, the H^+

-form of the resin was obtained. The resin was transferred to a large beaker, washed with 2-3 portions of 80% ethanol, and kept under 80% ethanol.

Preparation of the column. The column consisted of a 1 x 30 cm glass tube. In the lower end of the tube was soldered a perforated glass bottom. Below the perforated bottom, the glass tube was drawn to a curved thick-walled capillary tube, bent upwards and downwards in such a way as to have its bend reaching 10 cm above the level of the bottom, and the end of the capillary pointing downwards. In its upper part, the column had a spherical enlargement, ending with an opening in which was fitted a rubber stopper with glass tube and stop-cock. This tube was connected to an air-pressure system, obtained with the help of two bottles filled with water. This system could be used simultaneously for operating several columns. The height of adsorbent column was 8 cm; it was prepared by introducing into the glass column a slurry of resin in 80% ethanol. The resin was allowed to settle, and was washed with 15-20 ml of 80% ethanol at a rate of 1 cc/min.

Extraction of free amino acids. Twenty grams of flour or bread were extracted with three successive portions of distilled water at 0°. The total volume of water was 140 ml in the case of wheat flour, dough and bread, and rye dough and bread, and 180 ml in the case of rye flour. Each extraction lasted 10 min; it was carried out by triturating a weighted amount of the product with ice-cold water in a mortar cooled with ice. After each extraction the suspension produced was centrifuged at 2000 r/min for 3 min. The combined extracts were treated with twice their volume of 95% ethanol (three times their volume in the case of rye flour and bread), and left to stand overnight in the cold. The extracts obtained from wheat flour and bread were filtered through filter paper, while extracts of rye flour and bread were first centrifuged, and then, filtered. The filtrates were extracted, while triturating with a glass rod, with 2 ml of distilled water (10 ml in the case of rye flour) for 15 min at room temperature. When solution was difficult, a little ethanol was added, the volume of which was taken into account later during the following treatment with ethanol. The extract was treated with 5 times its volume of 95% ethanol, and the amino acids it contained were then fractionated on a column of KU-2 resin. A funnel with a folded filter paper was introduced in the upper end of the ion-exchange column, and the solution of amino acids prepared as described above was poured in the column through this filter. The funnel and flask were washed 5 times with 80% ethanol (total volume, 20 ml), the washings were passed through the column, followed by 15 ml of 80% ethanol. The next step was elution of the amino acids from the column.

Elution was carried out in succession with: 80 ml of 0.4 N NH_4OH in 80% ethanol, 30 ml of 80% ethanol, 30 ml of boiled distilled water, 30 ml of 4N NH_4OH, and 30 ml of water. All the eluates were combined together and evaporated in vacuum at 50-55° to dryness. The residue was dissolved in 2 ml of 10% aqueous isopropanol, and the solution kept in the cold. The volume of solution put on the chromatograms was 5-30 μl.

When determining the content of free amino acids in wheat flour, it is not necessary to purify the extracts on the ion-exchange resin.

RESULTS OF THE STUDY

In order to elucidate the role of free amino acids in the reaction of melanoidine formation in bread baking it was necessary to determine the dynamics of their contents, starting with flour and ending with baked bread.

Composition of free amino acids in flour. The content of free amino acids was determined in four samples of wheat and rye flour of normal quality, and 72% and 99% grades.

Wheat flour No. 1 of commercial kind, 72% grade had its gluten of normal quality, and an ash content of 0.67%; wheat flour No. 2, milled in the laboratory mill "Nagema" from wheat of "Mil'turum" variety, and of normal quality, was of grade 72% and had an ash content of 0.62%. Rye flour No. 1, obtained on the same mill from rye of "Vyatka" variety of normal quality, was of grade 70%, and had an ash content of 0.94%; rye flour No. 2 was commercial, of normal quality, its grade was 99% and ash content, 1.74%.

The results of the assays are given in Table 1.

Both in wheat and rye flour, aspartic and glutamic acid predominate. The content of other amino acids is noticeably lower. Together with the mentioned amino acids (Table 1), one could detect on the chromatograms spots due to asparagine, threonine, tryptophan, proline, and arginine. Proline was detected only after treating the chromatogram with isatin [5]. The total number of amino acids identified on the chromatograms was 18.

Change in the content of free amino acids during bread baking. The dynamics of the content of free amino acids during the preparation of wheat bread were studied with the flour sample No. 2. Bread was prepared by the

TABLE 1

Content of Free Amino Acids in Wheat and Rye Flour (mg% of dry weight)

Amino acids	Wheat flour		Rye flour	
	№ 1	№ 2	№ 1	№ 2
Aspartic	28,1	9,4	20,8	19,7
Glutamic	12,7	10,5	20,6	20,7
Serine	3,8	1,2	5,8	3,4
Glycine	3,7	2,3	7,4	4,5
Alanine	9,4	4,9	9,6	19,2
Glutamine	5,6	2,0	9,1	1,2
γ-Aminobutyric + tyrosine	6,6	3,3	8,7	8,3 *
Valine	4,1	2,0	4,4	8,2
Leucine + isoleucine	8,9	3,6	—	8,8
Phenylalanine	6,4	2,2	5,3	2,8
Threonine	—	2,4	—	5,8
Methionine	0	0	—	0,3

* Without tyrosine.

TABLE 2

Change in the Content of Free Amino Acids During the Preparation of Wheat Bread

Amino acids	mg% of the dry weight		
	flour	crumb	crust
Aspartic	9,4	6,9	3,5
Glutamic	10,6	11,6	2,2
Serine	1,2	1,1	0,6
Glycine	2,3	3,1	1,6
Threonine	2,4	4,4	1,8
Alanine	4,9	6,5	2,7
Glutamine	2,0	1,2	0,2
γ-Aminobutyric + tyrosine	3,3	7,9	2,8
Valine	2,0	1,6	0,6
Leucine + isoleucine	3.6	2,6	1,0

unleavened procedure using the following quantities: flour, 960 g; yeast, 30 g; salt, 15 g; water content in dough, 44%. Fermentation for 160 min at 30°; ripening at 35° till ready. Baking at 225° to production of normally colored crust.

The results of the assay of free amino acids during baking of wheat bread are gathered in Table 2.

The data obtained clearly indicate that during baking the crust, as compared to the crumb, undergoes a considerable depletion in free amino acids. This depletion occured with all the amino acids assayed, and was especially high in the case of glutamic acid. From the given data, it is clear that during the formation of bread crust, there occurs a rather intensive melanoidine formation, in the process of which free amino acids are rapidly spent by reacting with reducing sugars and giving melanoidines.

The dynamics of the content of free amino acids during the preparation of rye table bread by the loaf method were observed with a sample of rye ripening flour No. 2, of 99% grade. Bread was prepared with the following quantities: leaven, 300 g; flour, 475 g; salt, 9.5 g. The leaven was obtained from the bakery, and was renewed several times; moisture content, 50%; final acidity, 13-15°; temperature, 30°; moisture content dough, 51%, final acidity 10-11°; fermentation temperature, 30°; ripening temperature, 35°; fermentation time, 1.5 hr; ripening time, 1 hr. The results of the assay of free amino acids in this experiment are given in Table 3.

The data in this table show that during the preparation of rye dough the content of free amino acids is rather considerably increased.

Differences in the action of lactic acid bacteria and yeasts on rye and wheat dough, in the acidity of rye and wheat dough in the grade of flour, in the activity of the proteolytic enzymes of yeasts and lactic acid

TABLE 3

Change in the Content of Free Amino Acids During the Preparation of Table Rye Bread

Amino acids	mg% of the dry weight				Change in dough in % w.r. to flour	Change in crumb in % w.r. to dough
	flour	dough	crumb	crust		
Aspartic	19,7	41,5	34,8	19,2	+ 110,7	—16,1
Glutamic	20,7	49,6	45,0	16,6	+ 139,6	— 9,3
Serine	3,4	3,4	4,1	2,9	0,0	+20,6
Glycine	4,5	11,4	11,5	8,2	+ 153,3	+ 0,9
Threonine	5,8	15,8	16,2	11,1	+ 172,4	+ 2,5
Alanine	19,2	41,9	44,1	29,3	+ 118,2	+ 5,3
γ-Aminobutyric acid	8,3	16,6	19,3	8,5	+ 100,0	+16,3
Valine	8,2	28,9	33,1	16,8	+ 252,4	+14,5
Leucine + isoleucine	8,8	46,2	51,8	24,1	+ 425,0	+12,1
Phenylalanine	2,8	22,6	28,2	—	+ 707,1	+24,8
Methionine	0,3	5,6	+	0,0	+ 1766,7	—
Glutamine	1,2	0,03	0,0	0,0	—	—

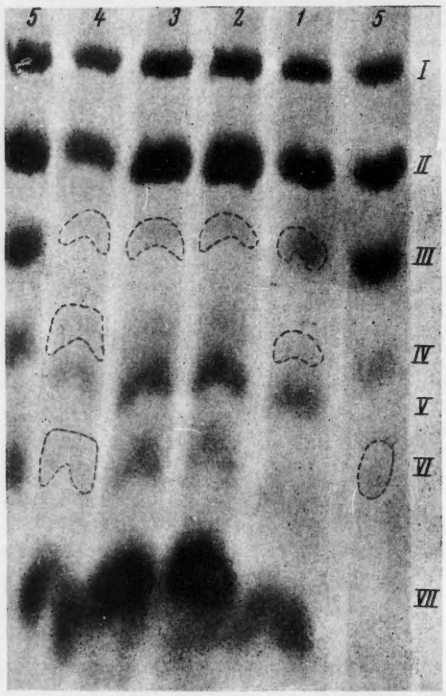

Fig. 1. Chromatogram of the free amino acids of table rye bread. Solvent: phenol–phosphate buffer, pH 12 (1:1). 1) Flour; 2) dough; 3) crumb; 4) crust; 5) controls. I–Aspartic acid; II–glutamic acid; III–serine; IV–glycine; V–asparagine; VI–threonine; VII–alanine+ glutamine.

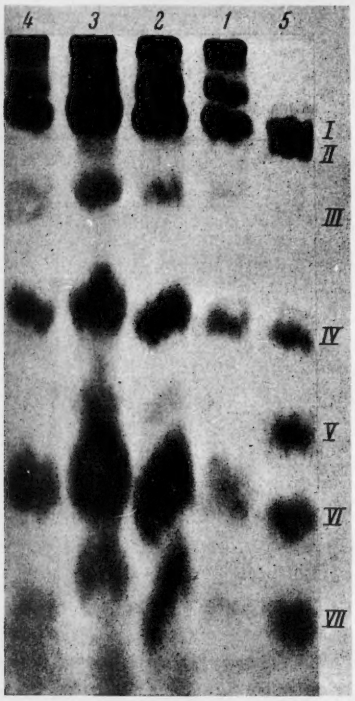

Fig. 2. Chromatogram of the free amino acids of rye bread prepared with addition of an enzyme preparation of Aspergillus oryzae. Solvent: o-cresol–phosphate buffer, pH 6.2 (1:1). 1) Flour; 2) dough; 3) crumb; 4) crust; 5) controls. I–Alanine; II–glutamine; III– γ-aminobutyric acid+ tyrosine; IV–valine; V–methionine; VI–leucine+ isoleucine; VII–phenylalanine.

bacteria–all these differences are probably responsible for the fact that the dynamics of free amino acids content during the preparation of wheat and rye dough are substantially different.

The influence of enzyme preparations of Aspergillus oryzae on the quality of table rye bread has been subjected to experimentation*. Such enzyme preparations are used in bread baking for the improvement of the quality of bread. The results of the assay of free amino acids in rye bread prepared without addition of the enzyme preparation are given in Table 3. Results obtained with bread made of the same flour, but with addition of the enzyme preparation are presented in Table 4. The two qualities of bread were prepared simultaneously. In the second case, the leaven was treated for a long period of time with 0.3% (w.r. to the weight of flour) of an enzyme preparation possessing amylolytic (400 units) and proteolytic (600 units) activities. Under the influence of the enzyme preparation, the ripening of leaven became more rapid, and the microflora was activated. The taste, aroma, and physical properties of bread prepared with such a leven were improved.

TABLE 4

Change in the Content of Free Amino Acids During the Preparation of Table Rye Bread Baked with the Addition of an Enzyme Preparation of Aspergillus oryzae

Amino acids	mg % of the dry weight				Change in dough in % w.r. to flour	Change in crumb in % w.r. to dough	Change in crumb in % to crumb of bread without enzyme
	flour	dough	crumb	crust			
Aspartic	19,7	14,9	55,9	14,6	— 24,4	+275,2	+ 60,6
Glutamic	20,7	39,0	54,7	11,6	+ 88,4	+ 40,3	+ 21,6
Serine	3,4	3,2	5,9	3,0	— 5,9	+ 84,4	+ 43,9
Glycine	4,5	11,3	17,2	7,1	+ 151,1	+ 52,2	+ 49,6
Threonine	5,8	23,5	34,1	11,0	+ 305,2	+ 45,1	+110,5
Alanine	19,2	38,0	58,9	24,8	+ 97,9	+ 55,0	+ 33,6
γ -Aminobutyric	8,3	17,2	22,7	7,7	+ 107,2	+ 32,0	+ 17,6
Valine	8,2	29,2	41,9	14,2	+ 256,1	+ 43,5	+ 26,6
Leucine + isoleucine	8,8	46,5	64,9	22,4	+ 428,4	+ 39,6	+ 25,3
Phenylalanine	2,8	25,2	41,0	11,0	+ 800,0	+ 62,7	+ 45,4
Methionine	0,3	8,0	—	0	+2666,7	—	—
Glutamine	1,2	Traces	Traces	Traces	—	—	—

The general trend of the change in free amino acids during the preparation of rye bread with addition of an enzyme preparation is analogous to that observed during the preparation of rye bread without added enzymes, namely, the content of free amino acids increases in dough in comparison with flour. However, it clearly appears that during the preparation of bread with added enzymes, the amount of free amino acids in the crumb is considerably higher than in the crumb of bread baked in the usual way.

It appears from the data of Tables 2 to 4 that in the crust of wheat, as well as that of rye bread, the content of all free amino acids without exception is sharply diminished. Thus, we succeeded to show by direct determinations the participation of free amino acids in the reaction of melanoidine formation occuring in bread crust during baking.

According to Tokareva's data [6], obtained in the same experiment in which we determined the dynamics of free amino acids content, addition of enzyme preparation provokes an intensive accumulation of reducing sugars in dough. The degree of participation of amino acids in melanoidine formation increases at the same time, being a consequence of the increased reducing sugars content in bread. This clearly appears in the calculations given in Table 5.

Thus, in bread crust without the enzyme preparation, the aspartic acid content decreases by 45%, and in bread crust with the enzyme, by 74%. The consumption of glycine, as a result of addition of enzyme preparation increases from 29% to 59%, and of alanine, from 34% to 58%. The fact that the decrease in amino acids content of the crusts parallels the increase in reducing sugars content in the crumb eloquently proves the most important role of the melanoidine formation reaction during the formation of bread crust.

The change in the content of free amino acids during the preparation of bread, viz., the increase of this content in dough, and its sharp decrease in crusts can be illustrated by the chromatograms of one of the experiments shown in Fig. 1 and 2.

*These experiments were carried out in collaboration with R. R. Tokareva of the Central Scientific Research Institute of Bread Baking Industry.

It is known that during the reaction of melanoidine formation, various aldehydes are produced at the same time as the darkly colored substances. Moreover, it was shown by Kretovich and Tokareva [1], that aldehydes are the substances responsible in the first place for the aroma of bread.

TABLE 5

Decrease in the Content of Amino Acids in Rye Bread Crust

Amino acids	Decrease in % w.r. to crumb		Amino acids	Decrease in % w.r. to crumb	
	with enzyme	without enzyme		with enzyme	without enzyme
Glutamic	79	63	Valine	66	49
Aspartic	74	45	Leucine+ isoleucine	65	53
Phenylalanine	73	–	Glycine	59	29
Threonine	68	31	Alanine	58	34
γ-Aminobutyric	66	56	Serine	49	29

In relation to this, one can note that the rye bread prepared in our experiments with the addition of enzyme preparation, and which had the best and strongest aroma, had, according to Tokareva, a higher aldehyde content. Thus, increased breaking down of free amino acids in bread crust, when bread was baked with addition of enzyme preparation, was accompanied with increased accumulation of aldehydes.

SUMMARY

A method was developed for a quantitative assay of free amino acids in flour, wheat and rye dough and bread. The method was based on extraction of free amino acids with ice-cold water, precipitation of the dissolved proteins, mucilages and dextrins with ethanol, separation of amino acids from sugars and salts on a KU-2 resin cation-exchange column, elution of the amino acids from the resin with alcoholic and aqueous ammonia solutions, concentration of the eluates containing amino acids in vacuum, and determination of amino acids by quantitative paper chromatography. In wheat and rye flour, aspartic and glutamic acids predominated. The change in content of free amino acids during the preparation of wheat and rye bread was followed with aspartic, and glutamic acids, serine, glycine, threonine, alanine, glutamine, γ-aminobutyric acid+ tyrosine, valine, leucine isoleucine, phenylalanine, and methionine. A considerable increase in free amino acids was found in ripe rye dough, as compared with the original flour.

A considerable loss of free amino acids occured in the crust of wheat and rye bread during baking. The loss of free amino acids in the crust increased with the increase of the amount of directly reducing sugars in the dough. Thus, the evidence obtained supports the participation of free amino acids in the reaction of melanoidine formation during bread baking.

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A STUDY OF THE ACTION OF COLLAGENASE ON SYNTHETIC SUBSTRATES

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In our previous communication [1] we showed that out of a large number of synthetic peptides and their derivatives we could find five substrates for collegenase isolated from Clostridium histolyticum. Somewhat earlier, communications had appeared from several laboratories [2-4] on the preparation of substrates which were very similar in composition to some of our synthetic ones, namely; carbobenzoxy (cbz)-alanylglycylproline NH_2 [2], cbz-prolylalanylglycylproline-NH_2 [2], cbz-hydroxyprolylglycylglycylproline [4], glycylprolylleucyl-glycylproline-NH_2 [3] poly(prolylleucylglycine), molec. wt. 3000 [3]. However, in none of these studies was there a detailed investigation of the products of the splitting of the synthetic substrates.

The collagenase substrates described in our previous work [1], which consisted exclusively of glycine and iminoacids, were of great interest because in the collagen molecule there is a considerable participation of polypeptide chains which contain only the above mentioned amino acids [5] and it is possible that it is just this portion which gives special specificity to the protein groups of collagen.

It is enough to say that such a tripeptide as glycylprolylhydroxyproline, contains about 40% of all the imino acids of the protein, and 20% of the imino acids are included in the tripeptide glycylprolylalanine [6]. Therefore, besides the study of the action of collagenase on peptides consisting of glycine, imino acids, and alanine, we studied its action also on peptides consisting only of glycine and imino acids (table).

Action of Collagenase on Some Derivative of Carbobenzoxypeptides

Compound number	Carbobenzoxypeptide derivative	Action of collagenase
1	Cbz-prolylalanylglycylproline-OCH_3	+
2	Cbz-prolylglycylglycylproline-OCH_3	–
3	Cbz-alanylglycylproline-OCH_3	–
4	Cbz-alanylglycylproline-NH_2	+
5	Cbz-prolylalanylglycine-OCH_3	–
6	Cbz-glycylprolylalanylglycine-OCH_3	–
7	Cbz-alanylglycylprolylproline-OCH_3	+
8	Cbz-alanylglycylprolylhydroxyproline-OCH_3	+
9	Cbz-(glycylprolylhydroxyproline)$_2$-OCH_3	+
10	Cbz-(glycylprolylproline)$_2$-OCH_3	–
11	Cbz-prolylglycylglycylglycylproline-OCH_3	+
12	Cbz-(glycylprolylhydroxyproline)-(glycylprolylproline)$_2$-OCH_3	Acts weakly
13	Cbz-(glycylprolylhydroxyproline)$_2$-(glycylprolylproline)$_2$-OCH_3	" 2
14	Cbz-glycylprolylhydroxyprolylglycylproline-OCH_3	+
15	Cbz-(glycylprolylproline)$_3$-OCH_3	Acts weakly

Note. Plus sign means collagenase acts; minus that it does not act.

Of the products of splitting of collagen by collagenase which have been studied it has been shown [2,7] that here the amino group of glycine is liberated almost exclusively. The carboxyl group is liberated by different amino acids; at the carboxyl end arginine, hydroxyproline, and alanine are found somewhat more often than other amino acids.

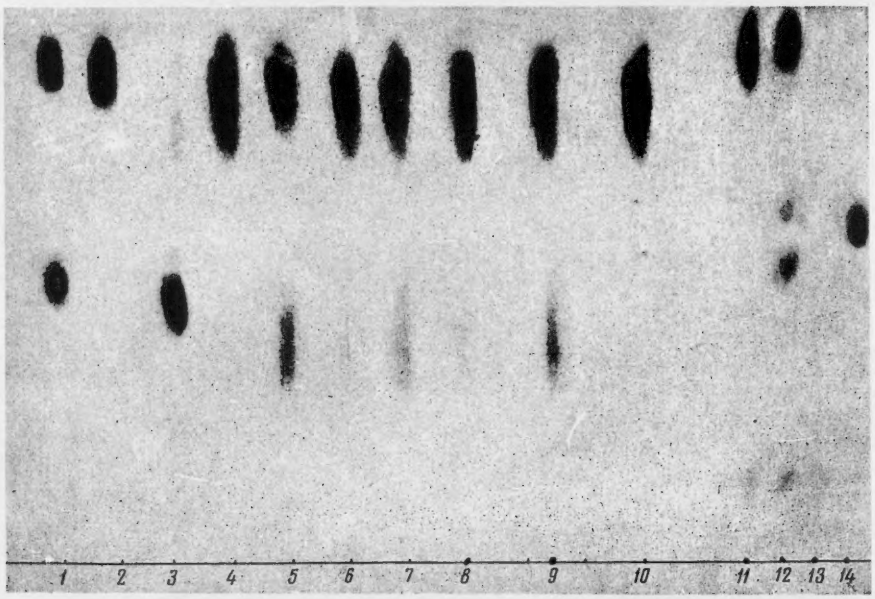
The purpose of the present work is to study the products of splitting of synthetic substrates of collagenase to establish the point of rupture of the peptide bond by the enzyme, and also to study some "model" peptide chains to explain the conditions which are sufficient for splitting the peptide bind by collagenase.

EXPERIMENTAL

We studied the splitting of the following collagenase substrates: amide of cbz-alanylglycylproline, methyl ester of cbz-prolylalanylglycylproline, methyl ester of cbz-prolylglycylglycylglycylproline, methyl ester of cbz-(glycylprolylhydroxyproline)$_2$, and we also demonstrated the action of collagenase on the methyl esters of cbz-glycylprolylhydroxyprolylglycylprolylprolylglycylprolylproline, cbz-(glycylprolylhydroxyproline)$_2$(glycylprolyl-proline)$_2$. cbz-alanylglycylprolylproline, and cbz-alanylglycylprolylhydroxyproline. We should note that our synthetic methyl esters of cbz-glycylalanylprolylglycine and cbz-prolylglycylglycylglycylproline were not split by the enzyme. Modeling of the peptide chains, in other words changes in the region around the peptide bond which is hydrolyzed by the enzyme, is shown in the table.

Enzymatic hydrolysis was carried out for all the substrates under standard conditions. Two to three mg of substrate was mixed with 40-60 μg of dry collagenase [7] and the mixture was treated with 0.25 ml of 3% $CaCl_2$ whose pH was first brought to 7 by addition of a solution of $Ca(OH)_2$. The solution was kept in a thermostat for twelve hours at 37° Control experiments were carried out under the same conditions; the composition of the control sample was: 1) 0.25 ml of 3% $CaCl_2$+ 40-60 μg of collagenase; 2) 0.25 ml of 3μ $CaCl_2$+ 2-3 mg of substrate.

Chromatography after enzymatic hydrolysis was always carried out in the system butanol-water-CH_3COOH (4:5:1) on "Leningrad B" paper. A typical chromatogram is given in the figure.



Chromatograms of Collagenase Hydrolyzates of peptides. Hydrolyzates. 1) cbz-L-prolyl-DL-alanylglycyl-L-proline-OCH_3; 3) cbz-glycylprolylhydroxyprolylglycylprolylhydroxyprolyl-OCH_3; cbz-(glycylprolylproline)$_2$-OCH_3; 12) cbz-clycylprolylhydroxyprolylglycylglycylpro-lyline-OCH_3; 2,4,6,8,10,11,13) corresponding standards; 14) cbz-prolylglycylglycylglycyl-proline-OCH_3.

Study of the Products of Enzymatic Splitting

Amide of cbz-alanylglycylproline. In chromatography of the enzymatic hydrolyzate and later development of the chromatogram with toluidine two spots were found, the first for a carbobenzoxy derivative with R_f 0.90, and the second with R_f 0.27. The second spot was eluted from the chromatograph paper and hydrolyzed by six hour boiling with 6 N HCl. The hydrolyzate was evaporated dry in a vacuum, dissolved in a small amount of water, and again evaporated. Chromatograph of the hydrolyzate showed the presence in it of glycine and proline which were identified by standards and by color with isatin.

Methyl ester of cbz-prolylalanylglycylproline. In the chromatogram from the enzymatic hydrolyzate we found two substances which corresponded to spots with R_f 0.9 and R_f 0.49. The second spot was eluted from the chromatogram and converted to the dinitrophenyl compound according to Sanger. The DNP-derivative of the methyl ester of the peptide was extracted with ether, the ether was distilled off, the residue was treated with 2 ml of 6 N HCl and hydrolysis was carried out in a sealed ampule for 12 hours at 105°. The acid hydrolyzate was extracted with ether, the ether was distilled off, and the residue was hydrolyzed with concentrated ammonia in a sealed ampule for four hours at 105°. The ammoniacal hydrolyzate was evaporated dry, the residue was dissolved in a small amount of water and chromatographed in the system butanol-water-CH_3COOH. In the hydrolyzate we found glycine which was identified by a standard.

Besides the dinitro phenyl treatment we carried out a complete acid hydrolysis of the second spot; in the hydrolyzate we found only glycine and proline.

Methyl ester of cbz-prolylglycylglycylglycylproline. In the chromatography of the enzymatic hydrolyzate of this pentapeptide we found two products with R_f 0.86 and R_f 0.54. The second spot was eluted from the chromatogram and after its complete hydrolysis was identified by standards in paper chromatography as the hydrolyzate glycine and proline. In coloring the chromatogram with isatin, the spot with R_f 0.54 was colored yellow which indicates glycine at the ammonia end of the peptide. Due to the weak action of the enzyme on this substrate we have not yet succeeded in carrying out a quantitative dinitrophenyl treatment for making the point of rupture of the bond more precise.

Methyl ester of cbz-glycylprolylhydroxyprolylglycylprolylhydroxyproline. In chromatography of the enzymatic hydrolyzate, we found two products by tolluidine development: one with R_f 0.38 and the other with R_f 0.86; the spot with R_f 0.38 was eluted and hydrolyzed as above. In the chromatogram we found glycine, proline, and hydroxyproline which were identified by standards and by isatin coloring. It should be said that in coloring the chromatogram of the enzymatic hydrolyzate with isatin, the spot with R_f 0.38 was colored yellow which indicates the presence of glycine on the amino end of the peptide.

Methyl ester of cbz-alanylglycylprolylhydroxyproline. In chromatography of the enzymatic hydrolyzate we found after toluidine development two products, with R_f 0.5 and R_f 0.9. When the chromatogram was colored with isatin it was observed that the product was yellow which indicates a rupture of the alanyl-glycyl bond; actually, in the case of a rupture of the glycyl-prolyl bond there would be formation of prolylhydroxyproline either as a dipeptide or its anhydride and the color of the spot with isatin should then be blue, which is not found in this case.

Methyl ester of cbz-alanylglycylprolylproline. A study of the products of hydrolysis was carried out in the same way as for the preceding derivatives; it was showed that splitting occurred at the alanyl-glycyl bond.

DISCUSSION OF RESULTS

In study of collagenase hydrolyzates of carbobenzoxypeptide methyl esters by paper chromatography with use in a number of cases of identification of the N-terminal amino acids by the dinitrophenyl method, and also by coloring the products of enzyme hydrolysis with isatin, we succeeded in showing that in compounds 1,4,7, 8 (table) the alanyl-glycyl bond is hydrolyzed; in compound 9 the hydroxyprolyl-glycine bond is hydrolyzed, and in compound 11 perhaps it is the prolyl-glycine bond (if we do not consider the possibility of splitting the glycyl-glycine bond by collagenase, although in the presence of two proline residues in the peptide this is not excluded).

From the results of our work and the data known from the literature we can observe from our point of view three conditions which are needed for the splitting of peptide bonds by collagenase.

1. It follows from the splitting of collagen by collagenase and from the structures of all the collagenase substrates obtained up to now that there should be amino groups of glycine and carboxyl groups of some other amino acid in the peptide bond which is split by collagenase. This conclusion is again confirmed in our work, since in all of our substrates there is rupture of the bond x-glycine (where x is alanine or hydroxyproline).

2. Although at the carboxyl end when procollagen is split by collagenase we often find almost all amino acids, yet it is likely that the nature of the amino acid x in splitting the bond x-glycine may play a decisive role. Actually we showed in our previous work [1] that the methyl ester of the peptide cbz-prolylglycylglycyl-proline is not split by collagenase, but the methyl ester of cbz-prolylalanylglycylproline is split.

3. Third and perhaps most important, evidently, is the essential condition for splitting the bond that there must be present along with this bond two, or sometimes one, proline according to the scheme prolyl-x-glycyl-proline, where, as the literature shows, x is any amino acid. However, this consideration must be accepted with care, since it is evident from the above examples that when x is replaced, stricter conditions must be imposed. It follows that proline after the glycine and connected with it by its own imino nitrogen plays a greater role in the splitting of the bond than does proline bound by its carboxyl group with x. For example, it has been shown that the amide of cbz-glycylalanylproline is split by collagenase while the amide of cbz-prolyalanylglycine is not split. The introduction of glycine between the carbobenzoxy group and proline, that is, obtaining the methyl ester of cbz-glycylprolylalanylglycine does not make it a substrate, and hence proline does not affect the splitting of the bond even when it stands in the ordinary peptide chain. Our observation is that for the action of collagenase it is not a matter of indifference what bond is on the carboxyl end of the proline which is bound by its imino nitrogen to glycine. Thus, the methyl ester of cbz-alanylglycylproline is not hydrolyzed by collagenase while the amide of this compound undergoes the action of this enzyme.

Since the second bond of proline can affect the splitting of the bond, we carried out "modeling" of the peptide chains of the substrates cbz-prolylalanylglycine and cbz-alanylglycylproline in order to place proline in these peptides in different locations. We showed that our synthetic methyl esters of cbz-alanylglycylprolyl-proline and cbz-alanylglycylprolylhydroxyproline are substrates for collagenase, while (as reported above) the methyl ester of cbz-glycylprolylalanylglycine is not split by the enzyme.

There is general interest in the fact which we established, that the methyl ester of the peptide cbz-(glycylprolylhydroxyproline)$_2$ is easily split by collagenase at the hydroxyprolyl-glycine bond and the ester of the longer peptides cbz-glycylprolylhydroxyprolyl(glycylprolylproline)$_2$ and cbz-(glycylprolylhydroxyprolyl)$_2$ (glycylprolylproline)$_2$ undergo the action of the enzyme to a much less degree. It seems possible to us to explain this fact by configurational changes in the polypeptide chain caused by the presence of a large number of imino acids in a comparatively short space, which could lead to inaccessibility of the hydroxyprolyl-glycine bond to collagenase.

The above experiments show indirectly that the peptide glycylprolylhydroxyproline which is obtained in large amount from collagen, is present in it in such a sequence that the quantity of imino acids as located in the collagen is less than in our peptides. In other words, for the action of collagenase, on the one hand we must have imino acids, and on the other, we must know their "dilution" by other amino acids. This conclusion agrees with our findings that the methyl ester of cbz-prolylglycylglycylglycylproline is not split by collagenase, while cbz-prolylglycylglycylglycylglycylproline undergoes the action of the enzyme.

It is important to note that collagenase splits not only the peptide bond between alanine and glycine in the substrates, but also splits the bond between imino acids and glycine. Here in a number of cases there appears a strong difference in the roles of proline and hydroxyproline. Thus, the methyl ester of cbz-glycylprolylhydroxy-prolylglycylprolylhydroxyproline is easily hydrolyzed by collagenase, while replacement in this compound of hydroxyproline by proline results in a compound upon which collagenase does not act.

The mechanism for hydrolyzing peptide bonds by collagenase suggested in the literature and the above ideas on the inequality of the two prolines apply only to substrates which have comparatively short chains. However, with growth of the polypeptide chain there will certainly appear new conditions, as a result of which earlier hydrolyzing peptide bonds will not be hydrolyzed by the enzyme, and vice versa.

SUMMARY

We have synthesized the methyl esters of cbz-glycylprolylhydroxyprolyl-(glycylprolylproline)$_2$, cbz-

(glycylprolylhydroxyproline)$_2$(glycylprolylproline)$_2$, cbz-glycylalanylprolylhydroxyproline, and cbz-glycylalanylprolylproline as substrates for collagenase.

We have studied the splitting by collagenase of five of the substrates which we synthesized previously: methyl esters of cbz-alanylglycylprolylproline, cbz-alanylglycylprolylhydroxyproline, cbz-prolylalanylglycylproline, cbz-(glycylprolylhydroxyproline)$_2$-OCH_3, and cbz-prolylglycylglycylglycylproline.

We have discussed the the characteristics of structure of peptides which are substrates for collagenase.

In conclusion, the authors thank Director of the Institute of Medical and Biological Chemistry, AMN, USSR, V. N. Orekhovich and G. A. Levdikov for supplying the collagenase.

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NUCLEOTIDE METABOLISM OF Gallfria mellonella L CATERPILLARS, NORMAL AND AFTER X-RAY IRRADIATION

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Original article submitted June 10, 1960

It was shown previously on bone marrow [1-3] that after irradiation there is a considerable decrease in amount of free nucleotides, especially in nucleosidepolyphosphates, on the next day after irradiation, and also there is inhibition of autolytic splitting of RNA in homogenates and extracts. Some authors [4-6] have noted after irradiation an activation of autolytic splitting of proteins.

In order to explain the general biological rules of radiation damage to nucleotide metabolism we have used in the present work the caterpillars of the bee moth. Below we give data on their content of acid soluble purine and pyrimidine compounds and also on autolysis under normal and radiation conditions.

METHODS

In the experiments we used caterpillars in the late stage of development with an average weight of about 0.2 g. Irradiation was carried out in a glass vessel with a diameter of 4.5 cm and a height of 3.0 cm covered with gauze or cellophane. The source of radiation was an RUP-1 tube. Conditions of irradiation: 15 ma, 210 kv, filter 0.5 mm Cu + 0.75 mm Al. Time of irradiation 5 min 40 sec, focus distance 15 cm, strength of dose 352 r/min, dose 2000 r. Nonirradiated caterpillars in the same stage of development were kept under the same conditions to serve as controls.

A day after irradiation the experimental and control caterpillars were weighed and transfered to a cold room and to each of them, in separate samples, was added 3.5 ml of 0.3 M orthophosphate buffer, pH 7.0, and they were homogenized. From the irradiated and nonirradiated caterpillars we took samples without incubation and with incubation for one hour or three hours in a thermostat at 37°. In the samples without incubation the proteins and nucleic acids were precipitated quickly after irradiation by addition in the cold of 3.5 ml of 1.2 N $HClO_4$. The incubated samples were also transfered to a cold room and $HClO_4$ was added to them. The precipitate of proteins and nucleic acids was filtered off and the filtrate was neutralized with concentrated KOH. The precipitate of $KClO_4$ was filtered off, the filtrate was diluted and studied spectrophotometrically (the phosphate buffer solution added to the sample permitted more complete removal of nucleotides from the tissue).

The purine and pyrimidine compounds were adsorbed from aliquots of the filtrate on activated charcoal, then eluted with 60% ethanol with 1% ammonia [9] and the eluates were evaporated in a current of air at a temperature not above 40°. The residue was placed on chromatographic paper. Chromatography was carried out by the descending method using Leningrad slow filter paper which had first been washed with 25% CHOOH. Length of chromatogram 45 cm. As a solvent we used the mixture 95% ethanol-anhydrous n-butanol-1 M acetate ammonia buffer solution with pH 3.7-3.8 in the ratio 4:1:2 (the solvent was passed twice) [7].

All the numerical results in the work are the average of three or more experiments.

RESULTS AND DISCUSSION

Preformed free purine and pyrimidine compounds. The total absorption spectrum of the phosphate perchloric acid extracts of the caterpillars is given in Fig. 1. Absorption in ultraviolet light of the phosphate-perchlorate extracts of the caterpillars depends chiefly on the purine and pyrimidine compounds. The total content of these compounds a day after irradiation with a dose of 2000 r fell by 7%.

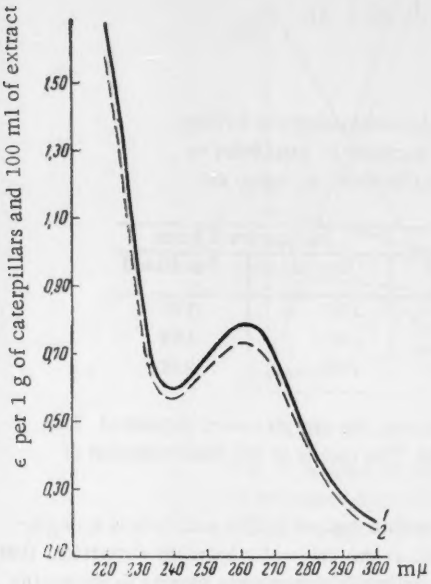

Fig. 1. Ultraviolet absorption spectrum of phosphate-perchlorate extracts of bee moth caterpillars : normal and a day after irradiation. Solid line-normal ; dotted line-after radiation.

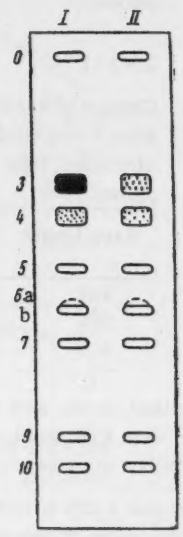

Fig. 2. Scheme of typical distribution on the chromatogram of acid soluble purine and pyrimidine compounds in normal caterpillars and those one day after irradiation with a dose of 2000 r. I) Normal; II) after irradiation. Figures to the left, fraction number; 0) starting position for the chromatogram; 3) ATP, 6) adenylic acid.

By the method described above we noted in the phosphate-perchloric acid extracts various low molecular weight substances with absorption maxima characteristic for nuclein bases, that is in the region 250-265 mμ. The position of these fractions on the chromatogram (Fig. 2) in most cases was characteristic for nucleotides and appeared very similar to the distribution of acid soluble purine and pyrimidine compounds of bone marrow when it was chromatographed under these conditions [2].

In parallel chromatography of the different fractions with markers in the form of different ribonucleotides it was shown that fraction 3 corresponded to ATP and fraction 6 to muscle adenylic acid.

The determination of the amount of purine and pyrimidine compounds contained in the phosphate-perchloric acid extracts of normal caterpillars and those a day after irradiation with a dose of 2000 r showed the following (the amount of the fraction is expressed in value of extinction at the maximum point, calculated per 1 g of caterpillar and 5 ml of eluate).

These results show that a day after irradiation the content in the caterpillars of different low molecular weight purines and pyrimidines is decreased in different ways. The ATP content decreased by 36%.

Autolysis of the caterpillars. Autolysis of the caterpillars was carried out in 0.3 M orthophosphate buffer solution at 37°. Controls were run in parallel without incubation. In each sample we took one caterpillar. The phosphate-perchloric acid extracts of purine and pyrimidine compounds in these experiments were obtained in

TABLE 1

Experimental conditions	Fraction number					
	3	4	5	6	7	9
Normal	0,644	0,303	0,073	0,043	0,028	0,032
Irradiated	0,414	0,201	0,047	0,036	0,023	0,028
Irradiated in % of normal	64	66	64	84	82	87

TABLE 2

Change in Autolysis of Bee Moth Caterpillars in Orthophosphate Buffers after X-ray Irradiation with a Dose of 2000 r. Increase in extinction in ultraviolet light of acid extracts; 24 hours after irradiation; value expressed in %

Wave length mμ	Incubation 1 hour		Incubation 3 hours	
	Normal	Irradiated	Normal	Irradiated
240	100	132	100	120
260	100	188	100	184
300	100	263	100	248

the same way as described above, with this difference, that after grinding, the samples were incubated. The filtrates after dilution were analyzed by absorption in ultraviolet light. The results of the determination of autolysis in the caterpillars are given in the table.

The table shows that a day after irradiation of the caterpillars with a dose of 2000 r autolysis is strengthened, judging by all the points of absorption in the ultraviolet studied. At the point of maximum extinction, that is, 260 mμ, the increase in autolysis is about 86%. The increase in autolysis is shown more clearly in determining the extinction at longer wave lengths. It is possible that this is connected with the fact that under the conditions of the experiment autolytic splitting occurs not only for the nucleic acids, but also for the proteins.

In both the normal and irradiated caterpillars (Fig. 3) the ratio of the different fractions in the process of autolysis changes in a similar way. In autolysis there is a sharp decrease in fraction 3, that is, in ATP. Fraction 4 which evidently corresponds to ADP decreases to a lesser extent under these conditions. Fraction 6, that is, adenylic acid, at first increases strongly and then lessens somewhat while fraction 7 increases for the duration of the whole experiment.

It is necessary to say that for bee moth caterpillars a radiation dose of 2000 r is not lethal and does not hinder their growth and metamorphosis. A dose of 25,000 r is also not lethal, though here inhibition of metamorphosis occurs. A dose of 100,000 r for the caterpillars is lethal in 15 days after the the irradiation.

The fact of the sharp fall in ATP content in the caterpillars after irradiation is analogous to the fall in ATP content reported in the literature for bone marrow [2] and for the spleen [8] after irradiation. The explanation of this general rule is evidently an inhibition of oxidative phosphorylation after irradiation. As already mentioned, a number of authors have noted a strengthening of autolysis of tissue proteins in higher animals after irradiation. In these studies orthophosphate buffers were not used and the autolysis was determined by the accumulation of amino groups of amino acids, but not by the extinction values in ultraviolet light which are characteristic for nucleotides.

The autolytic splitting of bone marrow RNA in orthophosphate buffer solution after X-ray irradiation in a dose of 2000 r is decreased 2-3 fold after seven hours and in later periods [3]. Marked activation of autolysis of caterpillars in the period immediately after irradiation may depend on the fact that after irradiation there is activation of different depolymerases of nucleic acids and proteins due to liberation of these enzymes from complexes which are breaking down.

Also, the initial activation of caterpillar autolysis is evidently closely connected with the inhibition of oxidative phosphorylation, which assures the biosynthesis of nucleosidepolyphosphates which then take part in the biosynthesis of nucleic acids and proteins. The inhibition of synthesis of nucleic acids and proteins should

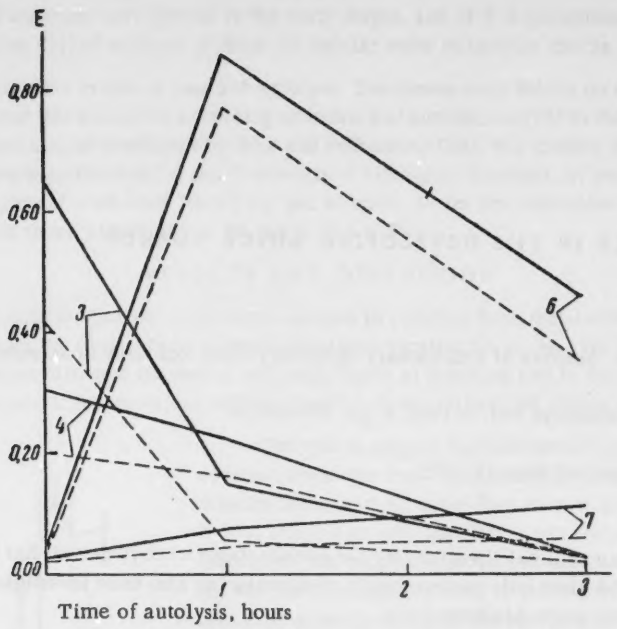

Fig. 3. Change in quantity of purine and pyrimidine fractions of bee moth caterpillars in autolysis of normal and irradiated subjects after irradiation with a dose of 2000 r. Amount of fraction expressed in value of extinction and at maximum point, calculated on 1 g of caterpillar and 5 ml of eluate. Figures on the curve denote fraction number. Solid line, normals, dotted line, after irradiation.

assist the appearance of the splitting process as is found in this case in the form of strengthened autolysis. As the amount of substrate is decreased by its easy splitting in autolysis and also in those cases when depolymerases may be liberated from the organism, the phase of activation of autolysis can shift to a phase of its inhibition.

SUMMARY

In the composition of the acid soluble fraction of bee moth caterpillars there is found adenosine triphosphate, adenylic acid, and other low molecular weight purine and pyrimidine compounds.

A day after X-ray irradiation of the caterpillars with a dose of 2000 r the total extinction of acid soluble compounds at 260 mμ decreases by 7%. The content of ATP falls by about 36%.

Autolysis of the caterpillars in orthophosphate buffer solution a day after irradiation with a dose of 2000 r increases by about 86%. A considerable fall in ATP content and activation of autolysis in the period immediately after irradiation can be explained by the inhibition of oxidative phosphorylation after irradiation.

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LACTIC ACID LEVELS IN THE DEVELOPING CHICK EMBRYO

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The role of glycolysis in animal tissues during the various stages of phylogenesis has been dealt with by a number of workers [1-3]. The glycolytic process during ontogenesis has also been investigated, but its study, particularly in the embryonal stage, has been scant.

Lactic acid formation in embryonal tissues was pointed out early by Bernard [4]. According to Warburg [5,6] and Needham [7], the anaerobic pathway of carbohydrate catabolism is characteristic for the early stages of ontogenetic development. The glycolytic acitivity of the embryonal tissues declines with the growth of the embryo. [8, 1]. Lactic acid was found in the chicken egg both before and after fertilization, and it was shown that its levels increased during the first five days of incubation, and fell thereafter [9, 10]. According to a number of authors [11, 12], the glycolysis within the chicken embryo itself was intensive in the initial stages, but fell with the increasing weight of the embryo. Novikov et al. [13] studied the glycolytic activity in homogenates of whole chicken embryos, 3 to 10 days old, and showed here also, an increase up to the fifth day, and a decrease thereafter. Drel' and Ilievskaya [14], on the other hand, showed a sharp increase in lactic acid content of chicken embryo during the tenth and twentieth day of incubation. Considerable glycolytic enzyme activity has been demonstrated in chicken eggs incubated as little as 12-24 hours [7].

Active aldolase has been demonstrated in the tissues of chicken embryos from the third day of incubation onwards [15, 16]. Considerable lactic dehydrogenase activity was demonstrated in the muscular and liver tissues of the chicken embryo during the sixth-to-eighth days of development [7, 17]. According to Solomon [17], the interval period between the ninth and 12th days of incubation are marked by very active synthesis of lactic dehydrogenase. Rashba [18] reported that chicken embryos contained adequate levels of glycolytic co-enzymes, some of which, for instance DPN and some pyrophosphates, were higher than in the postnatal period.

Unfortunately, most of the investigations dealing with glycolysis in chicken embryos were carried out with homogenates of the whole embryo, and not with individual tissues. Such treatment appears to be inadequate since, in adults at least, individual tissues are known to possess different levels of glycolytic activity. Of the published works dealing with lactic acid levels in embryos, only those of Rashba [19, 20] offered some information on individual tissue levels, but even those were confined to lactic acid levels in muscles in connection with their activity during hatching. Yet the study of lactic acid formation as a result of glycolysis, and as an indication of the balance between aerobic and anaerobic carbohydrate catabolism, is of considerable interest in the understanding of the carbohydrate metabolism in the embryonal tissues.

The aim of the present investigation was the determination of lactic acid levels in muscles, liver and blood of the chicken embryo during its development.

METHODS

White leghorn chicken embryos were used throughout. Lactic acid levels were determined in embryonal muscles (entire body muscle up to the 11th day of incubation, pectoral and leg muscles thereafter), in liver

tissue (the livers of 5-6 embryos were pooled in the early stages, and of 2-3 specimens later) and in blood drawn from the yolk-sac arteries [21] of embryos or from the jugular veins in hatched chicks.

The entire investigation involved over 300 embryos. The tissues were frozen on dry ice and transferred to cold 3% TCA. Lactic acid was estimated according to Barker and Summerson [22] in the TCA extracts. According to the above authors, and also as confirmed by Brba and Folbergova [23], this method is highly specific and sensitive, allowing the accurate estimation, in small amounts of biological material, of very low levels of lactate (from 3 μg, but in the present work even from 1 μg, per aliquot). Under the conditions of the present work the standard deviation of the mean values was in the range of ± 5-6%.

RESULTS AND DISCUSSION

Lactate levels in muscle. Lactate levels were assayed in embryos from the sixth day of development onwards. It can be seen from Fig. 1 that lactate levels remained constant up to he 16th day. Beginning with the 17th day the levels rose steadily and reached a very high figure at hatching and in the 12-24 hours immediately following hatching. Lactate levels remained high in muscles of newly-hatched chicks in the first 2-4 days.

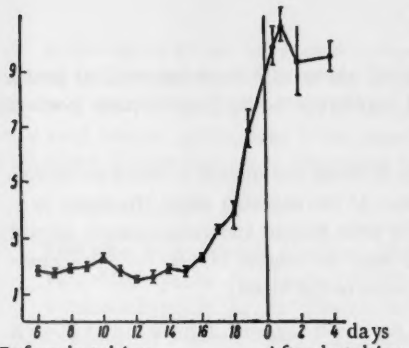

Fig. 1. Lactic acid content (μg per g wet weight) of the muscles of developing chicken embryos. (Here, and in Fig. 2, the vertical dashes on the curves delineate the extent of the standard deviation of the mean).

The results suggest that the rate of glycolysis remained fairly constant while the muscular tissue was being formed and while the muscles underwent no more than an occassional twitch or contraction. It is of interest to note that lactic dehydrogenase activity in the muscles of the chicken embryo also showed a certain constancy during the developmental period [17]. However, with the onset of more intensive muscular activity the rate of glycolysis increases sharply, resulting in an accumulation of lactate. Rashba [19] found increased lactate formation in the neck muscles during the hatching period (19-20 days), at a time when lactate formation in leg muscles was still low. The author explained this phenomenon by the specifically increased muscular activity of the neck in the process of hatching. In the present experiments a sharp increase in lactate accumulation during hatching was also observed in the leg muscles, thus indicating a high degree of activity also in the muscles of the legs. Indeed, it would be difficult to imagine that the complex and laborious task of breaking the shell and emerging from it could be accomplished without the participation of the muscles of the extremities.

The high lactate content of the leg muscles in the hatched chick observed in the present work was undoubtedly due to the newly acquired mobility. According to Rashba [19]the lactate levels in the leg muscles of young chicks (13.8 μ moles/g) were close to those encountered in corresponding muscles of adult birds (19.6 μmoles/g).

Another confirmation of the intensified glycolytic activity in the pre-hatching embryonal muscle comes from the observation that glycogen levels in the muscles of the embryo at the hatching stage and in the hatched chick (in the first 2-3 days) decrease sharply [20-24]. According to a number of authors [9, 20] glycogen is the main glycolytic substrate of the muscle of embryos in the second half of the incubation period. On the other hand, in the early stages of development glucose is the main substrate of glycolysis while glycogen, unlike in adult individuals, is not utilized. Glucose is, apparently, utilized together with glycogen in the later stages. Needham [7] pointed out that glucose was still the preferred substrate for glycolysis in the leg muscles of the 15-day old chick embryo. The observed increase in blood-sugar levels at hatching [21, 25] can probably be considered as an adaptive compensatory reaction aiming at increasing the glucose supply at the expense of liver glycogen. Indeed, glycogen levels in the livers have been shown to fall at the time of hatching [26, 27].

Regarding the further fate of the muscle lactate, Rashba [19] expressed the opinion that, since blood lactate levels remained unchanged during hatching, this compound was not likely to be transported to the liver, but was further metabolized in the muscle itself.

In view of this it was of interest to compare the lactate levels in muscle, blood and liver at the time of hatching.

Lactate levels in the blood. Blood lactate levels in the chicken embryo were found to fluctuate within narrow limits – from 1 to 2 μ moles/ml – during the incubation period (Fig. 2), and were thus quite close to those of muscle. At the hatching stage blood levels increased markedly, – an observation which is not in agreement with the results of Rashba [19]. Lactate levels in the blood rose to a maximum in 12-hour old chicks and thereafter decreased over the following 3-4 days until the previous lower levels were reached.

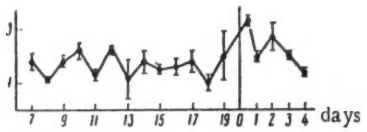

Fig. 2. Blood lactate levels (in μ moles / ml) in the developing chicken embryo.

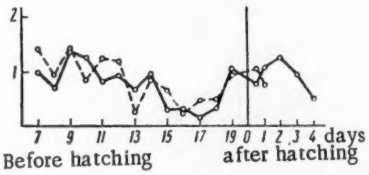

Fig. 3. Liver lactate levels (in μ moles / g wet tissue) in the developing chicken embryo.

A comparison of the blood lactate levels of chicken embryos reported above with those reported for young chickens and adult roosters [27] showed that, even in the early stages of development, the blood lactate levels of chicken embryos did not differ appreciably from those of adult birds.

The agreement between the two curves representing lactate levels in blood and muscle of chick embryos, respectively, indicates that muscle is probably the source of blood lactate. At the hatching stage glycolysis in muscle increases many-fold, with the result that large amounts of lactate were formed and accumulated. Regardless of the increased volume of blood-flow, the lactate was not removed from the muscle rapidly enough, consequently at that stage lactate levels in muscle were greatly in excess of those in the blood.

Lactate levels in the liver. During the initial stages of incubation (up to 10 days–cf. Fig. 3) lactate levels in the liver remained of the same order as those in muscle and blood. From the tenth day onwards liver lactate levels decreased steadily, reaching minimal values about the 15th-18th day, and then increased again, particularly during hatching. The high levels of liver lactate observed during hatching persisted for about three days following hatching, and then fell again.

A comparison between the lactate levels in liver and blood suggests that liver lactate apparently has its origin in blood, whose lactate in turn comes from the muscle pool. However, local reactions in the liver also enter the picture and endogenously produced lactate is probably superimposed on that entering from the bloodstream. It is of interest to note that the embryonal liver shows considerably more intense glycolytic activity than does adult liver. [8]. Indeed, lactate levels in liver were high up to the tenth day of incubation. During this period (7th-12th day), as shown by Solomon [17], a sharp increase (up to fivefold) in the lactic dehydrogenase activity took place in the chick embryo liver, followed by a decrease later.

During this period oxidative processes apparently take place in the liver together with glycolysis. The existence of the tricarboxylic acid cycle was demonstrated in livers of 5-day old embryos by Carey and Greville [28], but the activity of the cycle enzymes was still very low. Oxidative processes in the liver gained intensity after the tenth day. This was suggested by the demonstration of considerable activity of a number of the tricarboxylic acid cycle enzymes–succinic, α-ketoglutaric and pyruvic dehydrogenases, as well as fumarase and aconitase–in homogenates of livers taken from 10-day old chicken embryos [29]. It is thus possible that the observed decrease of lactate levels in livers of embryos between the 13th and 18th days was due to the increased levels of oxidative processes. It was also shown by Leibson [25] that intensive deposition of glycogen commences in the embryonal liver after the 13th day of incubation. In this process lactate can be utilized as a starting material [10].

Approximately in the same period there is considerable intensification of the precesses of differentiation in the embryonal liver and this, according to Solomon [17], is accompanied by increased activity of a number of enzymes. The latter author considers the 15th day of development to be a critical point, where the liver enters a phase similar functionally to the state of the adult liver.

The increase in lactate levels in the liver starting from the 19th day is most probably due to the increased flow of this compound from the muscle, through the bloodstream, and the inability of the liver to deal with the lactate at a rate compatible with its influx.

It thus follows from the comparative data presented above that the main source of lactate in the embryo (apart from the central nervous system, which was not included in the present study) is the muscular tissue. A portion of the lactate formed in the muscle passes into the bloodstream and, eventually, finds its way into the liver. Some portion of the lactate is, however, metabolized locally in the muscle, most probably by way of glycogen synthesis. It is probably oxidized only to a slight extent. According to Hall [30], lactic acid does not support respiration in muscular tissue in vitro, but leads to increased glycogen deposition.

The general impression formed in the study of lactate distribution in he chick embryo is that lactate levels were uniform in various tissues metabolically as far apart as liver and muscle, until those tissues differentiated and acquired their specific structures and the specific functions characteristic for each in the adult animal. Thus, in the later stages of development the pattern of carbohydrate catabolism in muscle tended towards glycolysis, which in this tissue is the main energetic process directly connected with its activity, while in liver the oxidative pathway predominated.

SUMMARY

In the tissues of the chick embryo during the first half of the incubation period rather constant and similar levels of lactic acid were found in the muscles, liver and blood. In the liver, the lactate level decreases drastically from the 15th to the 18th day of development. The hatching period is accompanied by an increase in the lactic acid content, particulary in the muscles, and less so in the liver and blood. The relationship of glycolysis to oxidative transformations in embryonic tissues is discussed.

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A STUDY OF THE ACCUMULATION AND TRANSFORMATION OF PROTOCHLOROPHYLLIDE IN THE GREEN PLANT BY THE METHOD OF RADIOCHROMATOGRAPHY WITH A CARRIER

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In recent years the idea of protochlorophyll as a percursor of chlorophyll in illuminated etiolated leaves has undergone several changes. It has been shown that etiolated shoots contain not only protochlorophyll, but also its form lacking phytol, protochlorophyllide. A number of results have been obtained showing that protochlorophyllide is hydrogenated faster than protochlorophyll when illuminated, giving the source for the phorbin nucleus of chlorophyll.

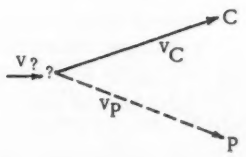
Considerable success has also been attained in solving the question of the synthesis of chlorophyll in green leaves. A single report, not accompanied by experiment data [1], on the presence of protochlorophyll pigment in illuminated green leaves was recently given in the literature. Litvin et al. [2] showed that the maximum fluorescence of protochlorophyll in green leaves in the light rose on transfer of the plants to the dark and weakened again with a second illumination. According to the results of the author the fluorescence of protochlorophyll in green leaves depended chiefly on the active form of the pigment.

We have isolated from green leaves in the light a pigment identified as protochlorophyllide [3]. It was shown that its content increased after transfer of the plant to the dark and decreased when it was illumined at low temperature [4], which agreed with the idea of the pigment as an intermediate product of chlorophyll formation.

Litvin, Krasnovskii, and Rikhereva [2] observed that the rate of accumulation of the pigment with the fluorescence spectrum of protochlorophyll in the dark corresponded with our data on the rate of renewal of chlorophyll in the light. The authors showed correctly that this is an argument in favor of the scheme of biosynthesis of chlorophyll (C) through the protochlorophyll pigment (P):

$$\xrightarrow{v_P} P \xrightarrow{v_C} C.$$

Under stationary conditions the rates of the reactions v_P and v_C are equal and the usual equation indicates that in dark blocking of the transformation P→C, accumulation of P should begin at a rate $v_P = v_C$, which normally characterizes the rate of formation of C. However, it is proper to emphasize that the analogous case can be complicated when the normal chain of biosynthesis does not block P, but in blocking the normal reaction it can arise from some sort of percursor (?).



If in the normal case $v_? = v_C$, and in the dark instead of C, P begins to accumulate, then under the conditions in which the limiting step of the total rate is the formation of the intermediate product, there will properly be the equation $v_P = v_?$ and hence, $v_P = v_C$, that is, the initial rate of accumulation of P will equal the normally observed rate of formation of C. Hence the limited character of similar arguments become evident.

In the present work we attempt to use the method of radiochromatography with a carrier to study the dynamics of accumulation and transformation of these pigments.

METHODS

Green barley plants grown under laboratory conditions with illumination of about 5000 lux, 6-8 days old, were placed for 40 min in a chamber which contained $C^{14}O_2$. The chamber was a glass vessel inverted over mercury and illuminated with nine luminescent lamps DS-15 placed at a distance of 30-50 cm from the plants. The illumination in the chamber was 3000 lux. At the end of the exposure the plants were placed for 24 hours in the dark for accumulation in them of a quantity of protochlorophyll pigments in addition to those gained in the light.

Samples were fixed in the dark by treatment for 2 min with hot steam. Then they were extracted with 80% acetone and in the usual way the pigment was transfered to petroleum ether. As we had shown previously [3] here the protochlorophyllide remained in the water-acetone layer and the protochlorophyll should pass into the petroleum ether. In agreement with this, further analysis of each layer was carried out separately. The water-acetone layer was treated with benzene which extracted the protochlorophyllide from it, and the concentration of the pigment was determined from the absorption spectrum. The content of the pigment was insufficient for purification by rechromatography which involves a marked loss, and therefore, to the extract obtained from the labeled material we added as a carrier 11.8 times more protochlorophyllide isolated in a similar way from unlabeled green barley plants (HCl number 12).

Purification of the protochlorophyllide was carried out by five fold rechromatography. After the first, third, and fifth rechromatography we determined the specific activity (SA) of the pigment, for which we prepared an extremely fine sample which did not require correction for self absorption [5]. The measured activity value was related to the amount of chlorophyllide in the preparation determined spectrophotometrically [6] on an SF-5 apparatus. In the calculation we used the equation for the molar extinction coefficient of protochlorophyllide and protochlorophyll of etiolated sprouts.

RESULTS AND DISCUSSION

The results of the determination of specific activity (SA) of protochlorophyllide are given in Table 1.

TABLE 1

Specific Activity of Isolated Protochlorophyllide

Numer of chromatographs	Composition of solvent	Number of preparation	SA of protochlorophyllide, counts/min/μg C
1	Petroleum ether-acetone-isopropanol (5:1:0.2)	1 2	1013 1137 } 1075
2	Benzene-petroleum ether-acetone (3:1:0.1)	–	Not determined
3	Ethanol-petroleum ether (1:7)	1 2	1092 1118 } 1105
4	Benzine-petroleum ether-acetone-isopropanol (10:2.5:2:0;5)	–	Not determined
5	Benzine-petroleum ether-acetone-methanol-isopropanol (10:2.5:1:0.25:0.5)	1	1108

Table 1 shows that the SA during the process of multiple rechromatography remains practically constant. Such constancy is retained even though the amount of pigment in the preparations obtained after each chromatographic run is almost halved because of the rejected portions, and amounts over the whole run to 4% loss. Thus

there is a parallel proportional decrease in radioactive material. This is convincing evidence that the resulting value is actually the SA of the chromatographically pure pigment, not distorted by any impurities. If we consider that at the beginning of the analysis of protochlorophyllide the experimental samples were diluted 12.8 times with carrier, the SA of the carbon in the pigment in the tissues is 14180 counts/min/μg C.

The exposure selected lies on the ascending branch of the curve of SA for chlorophyll and when illumination is continued even the plant removed from the chamber with the $C^{14}O_2$ should show further rise in concentration of C^{14} in the chlorophyll. In our other experiments with different plants the maximum SA of chlorophyll a was observed only several days after assimilation of C^{14} [7]. This means that during this time the SA of the precursor carbon remains even higher than in the chlorophyll itself, and only later does this relation become reversed. Therefore, along with the protochlorophyllide from the experimental sample we also isolated the chlorophyll a, the SA of whose carbon was equal to 1100 counts/min/μg C. Thus, the SA of the protochlorophyllide carbon under the conditions of the experiment was 12.9 times higher than that of the carbon of chlorophyll a. In agreement with the usually observed relation [8], the SA of the carbon of chlorophyll b in this experiment was much less than in chlorophyll a, and equalled 247.5 counts/min/μg C. Thus, as compared to chlorophyll b, the SA of the protochlorophyllide carbon was 57.3 times greater. However, it is more correct to compare protochlorophyllide not with the molecule of chlorophyll as a whole, but only with its phorbin part. For such a comparison we split the chlorophyll preparation with 15% NaOH in methanol and determined the radioactivity of each product separately.

As the results showed, the SA of the carbon from the phorbin part of the chlorophyll a was 895, and from chlorophyll b, 225 counts/min/μg C, which is 16 and 63 times less than the SA of the protochlorophyllide. The considerably higher SA of protochlorophyllide than of chlorophyll a confirms the idea of a role of this pigment as a precursor of chlorophyll. The high SA of protochlorophyllide is also explained by the fact that though the concentration of this pigment in the tissues is only 1% of the concentration of chlorophyll a the content of C^{14} in it reaches 12% of the content of C^{14} in chlorophyll a.

It should be noted that the constancy of SA of protochlorophyllide in the rechromatograpy distinguishes its purification from that of chlorophyll. In the case of chlorophyll it has been noted [9] that is is difficult to remove small amounts of impurities with high SA which, without influencing the spectrum, actually raise the initial activity ascribed to the pigment. Evidently protochlorophyllide is free from analogous impurities and even if its preparations contain any side substances, they do not have an increased SA and their presence in small amounts does not affect the results of the determinations.

From a consideration of the above results on the relation of SA values of protochlorophyllide and chlorophyll we are fully justified in assuming that it is just with the protochlorophyllide or substances analogous to it we are dealing with in the purification of chlorophyll. At the same time, the removal of considerable amounts of chlorophyll from preparations of protochlorophyllide should have a reverse action and lead to a constantly increasing SA. Such a result is actually found in one of the experiments, when for the isolation of protochlorophyllide we used its benzene solution and the ether extract which also contained chlorophyll, and rechromatographing was carried out in a less polar mixture of solvents.

For an explanation of the question of the presence of a neutral form of protochlorophyll in the green leaf to the pigment which was transfered from the acetone extract into petroleum ether we added as a carrier a chromatographically purified protochlorophyll from etiolated barley sprouts (fraction with HCl number 22) in an amount equal to the weight of chlorophyll a in the sample. The pigment mixture was then submitted to repeated chromatographic separation by the method worked out for purification of chlorophyll pigments [9].

The results of measurement of the SA are given in Table 2.

As Table 2 shows, as the protochlorophyll is purified, its SA falls very sharply and its value does not become constant in this experiment. Further treatment was impossible because of the decreasing amount of pigment. Therefore it is difficult to draw definite conclusions about the ability of green leaves to form a neutral form of protochlorophyll and it is possible to judge only the upper limits.

In agreement with these ideas, labeled protochlorophyll can be formed during the experiment through protochlorophyllide. Hence, if we take the total activity of the protochlorophyll, which is equal to that produced by the amount of carrier used (this greatly surpasses the content of endogenous protochlorophyll), and relate this in

TABLE 2

Change in Specific Activity of Protochlorophyll during Purification

Number of purifications	Composition of solvents	Number of preparations	SA of protochlorophyll, counts/min/μg C
1	Petroleum ether-ether-ethanol (30:10:0.5)	1 2	524 429 } 492
2	Benzene-petroleum ether-acetone (30:10:1)	–	Not determined
3	Ethyl alcohol-petroleum ether (1:7)	1 2 3	95 98 96 } 96.3
4	Benzine-petroluem ether-acetone (10:2.5:2)	–	Not determined
5	Benzine-petroleum ether-acetone-methanol (10:2.5:1:0.25)	1	8.9

terms of the SA of the pigment after its chromatography to the SA of protochlorophyllide in the tissues, and if we consider also the weight of the corresponding amount of phytol, we can find the content of of protochlorphyll in the sample which is formed after assimilation of C^{14} by the plant. Here, since the SA value found for protochlorophyll is not confirmed by continuing further its chromatography, but is rather the upper limit of its possible value, then the value calculated for the concentration of labeled protochlorophyll is also its upper limit. The orienting character of the results of the calculation as an upper limit is still further emphasized by the possibility of the presence of part of the C^{14} of protochlorophyll in its phytol, though we could not settle this by saponification of the sample because of its very slight radioactivity. The rather unsatisfactory nature of the calculation is shown by the fact that it is based on a single determination of the radioactivity of the protochlorophyllide and not on the kinetic curve of its transformation. The upper limit of content of labeled molecules of protochlorophyll found in this way, according to our results, is not more than 0.1% of the concentration of chlorophyll a, and hence its content of protochlorophyllide. However, these results still do not mean that the neutral form of protochlorophyll was, in general, present in our plants. The question of its presence in illuminated green leaves remains even more open. On the other hand, this also does not exclude the possibility of existence in them, apart form labeled protochlorophyll molecules, of some further quantity of pigment of earlier origin.

TABLE 3

Relative Radioactivity of Protochlorophyll Pigments. The data of all the experiments relates to amounts of plant material which contain 1 μg of chlorophyll a

Experiment No.	Experimental conditions	Carrier	Amount of carrier, μg	Values			
				A**	B**	C**	D**
I	Exposure in $C^{14}O_2$ 40 min, then 24 hours in the dark	Protochlorophyllide of green barley	0.118 (+0.010)*	1108	97	1100	0.119
II	The same	Protochlorophyllide of etiolated barley	1.0	8.9	6.6	1100	0.008
III	" "	Protochlorophyllide of etiolated barley	0.244 (+0.010)*	624	108	1100	0.133
IV	Exposure in $C^{14}O_2$ 25 min, then 24 hours in the dark	The same	6.85	1.7	8.0	115	0.094
V	The same + 3.5 min illumination	" "	0.685	1.9	0.9	159	0.008

* Content of endogenous protochlorophyllide in experimental sample

** Values A) SA of isolated pigment, counts/min/μg C; B) Total activity of pigment, counts/min; C) D) Ratio of total activity of pigments and of chlorophyll a.

We should note that in the experiments with protochlorophyllide the estimation of the total activity of the pigment in the sample was based on the idea that it passed completely into the benzene extract. However, it is evident that some of it could pass from the acetone to the petroleum ether and some portion could remain in the water-acetone layer in the extraction with benzene. In such a case the total activity of the protochlorophyllide would actually be higher than calculated and the relative content of protochlorophyll as compared to protochlorophyllide would be still lower. For a test of this possibility a special experiment was run as follows. Part of the labeled plants which were used in previous experiments was extracted with acetone and the pigments were then passed into ether. The water-acetone layer was treated with benzene and the pigments of the ether and benzene extracts were combined. Then as a carrier we added protochlorophyllide from etiolated sprouts (fraction with HCl number 12) and chromatographed successively five times in the above described mixtures of solvents. The resulting SA after being increased by the content of the mixture of pigment and carrier in the starting solution gave the value of the total activity of protochlorophyllide in the tissues, which in table 3 is compared with the results of the above experiments. As is seen, the total activity of protochlorophyllide in this case (experiment III) is about 10% higher than the value obtained before (experiment I). We can assume that just this portion of the protochlorophyllide is lost in the benzene extraction as described above. However, it is not impossible that there is an error in the determination, the more so that a definite constancy of protochlorophyllide SA was not reached in experiment III as had been planned. The great similarity in value of total activity of the pigments found in experiment I where the carrier was protochlorophyllide of green leaves and in experiment III with protochlorophyllide of etiolated sprouts is further evidence of the identity of protochlorophyllide of both sources.

Comparing the methods used for isolation of labeled protochlorophyllide in experiments I and III we can conclude that each of them is suitable for this purpose. However, a deficiency of the latter method is that we still do not know the concentration of protochlorophyllide in the tissues, and also the coefficient of dilution by the carrier, and therefore cannot calculate the SA of the pigment in the tissues. In experiment III this is done only because we used in it plants with a content of protochlorophyllide known from experiment I. Considering the coefficient of dilution by the carrier, in this experiment we get the SA of the carbon of protochlorophyllide in the tissues as 15,850 counts/min/μg C, which is sufficiently close to the value found in experiment I.

Experiments IV and V (table 3) were run like experiment III in order to establish the effect of repeated illumination on the content of labeled protochlorophyllide. For this purpose, part of the green barley plants illuminated for 25 min in the above chamber with $C^{14}O_2$ and then kept 24 hours in the dark, were illuminated for 3.5 min with diffuse daylight (4000 lux) and then fixed and treated by the method described, using as a carrier protochlorophyllide from etiolated barley sprouts (fraction with HCl number 12). In an attempt to reach a more definite constancy for the SA of the pigment, chromatography in this case was repeated seven times in succession. The results (experiment V) are shown in table 3 with the date of the control experiment run without repeated illumination (experiment IV).

Such a comparison clearly shows that even after 3.5 min illumination almost all the labeled protochlorophyllide formed in the period of darkness is transformed into chlorophyll and only 11% of it is still retained. If after darkness the content of C^{14} in the protochlorophyllide is 9.4% of the amount of C^{14} in the chlorophyll, which in spite of the difference in exposure agrees well with the results of experiments I and III, then after illumination this value fall to 0.8%.

The disappearance of protochlorophyllide is accompanied by an actual increase in specific and total activity of chlorophyll. This means that formation of new molecules of the latter occurs from materials with a higher content of labeled carbon. In the light of all the data obtained in our work these materials must be protochlorophyllide itself.

SUMMARY

It is shown that young green barley plants, transfered after short (25-40 min) assimilation of $C^{14}O_2$ to darkness for 24 hours contain protochlorophyllide in amounts reaching 1% of the content of chlorophyll a. The upper limit of content of labeled protochlorophyll is ten times less. The specific radioactivity of protochlorophyllide carbon is 16 and 63 times higher than that of the carbon of the phorbin part of the chlorophyll a and chlorophyll b, respectively. The total content of C^{14} in photochlorophyllide under these conditions reaches one tenth the radioactivity of chlorophyll a. Short illumination leads to practically complete (90%) disappearance of labeled protochlorophyllide with simultaneous increase in specific and total activity of chlorophyll a. The observed regularities agree with the idea of protochlorolhyllide as a normal precursor of chlorophyll in green leaves.

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THE KINETICS OF HYDROLYSIS OF SODIUM PHENYLPHOSPHATE UNDER THE INFLUENCE OF ALKALINE PHOSPHATASE

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There is a large and interesting group of experimental material [1-4] on the kinetics of the hydrolysis of phosphate esters under the influence of alkaline phosphatase. However, until now there have not been found kinetic equations which would describe with sufficient exactness the experimental regularities. In the present work we attempt to solve this problem.

To test our equations we have made a study of the relation of the rate of enzymatic hydrolysis of phenylphosphate to concentration of the enzyme and the concentration of the substrate.

METHOD

A crystalline preparation of alkaline phosphatase was obtained from dog intestinal mucosa by the method of Morton [5]. Ten mg of the enzyme was dissolved in 50 ml of distilled water. The resulting starting solution was kept in the refrigerator at 3-5°. The activity of the enzyme in this solution remained constant over a long period. Before beginning an experiment usually 1 ml of the starting solution was diluted to the desired concentration with distilled water.

The crystalline phenylphosphate used in the experiments contained practically no impurities: in its complete hydrolysis we found by the use of the analytical methods described below, phenol and sodium hydrogen phosphate in amounts which agreed exactly with the calculated values. Phenylphosphate was always dissolved in a carbonate buffer, pH 10 (6.36 g of anhydrous Na_2CO_3 and 3.36 g of $NaHCO_3$ in 1 liter of solution). This solution was used only on the day of the experiment.

In a water thermostat at 37° we placed a tube with 5 ml of solution of enzyme and a tube with a solution of phenylphosphate. After heating for 10 min, 5 ml of phenylphosphate solution was poured into the solution of the enzyme and from this moment the time was counted in seconds. Since the phenylphosphate was dissolved in a buffer solution, the value of the pH remained constant throughout the entire experiment which sometimes lasted more than a day.

The concentrations of phenol and sodium hydrogen phosphate formed in the enzymatic hydrolysis of phenylphosphate were determined by direct analysis of the samples which were taken from the reaction mixture at definite time intervals. To stop the enzymatic reaction of a given sample it was mixed directly with the corresponding analytical reagents. The phenol concentration was determined by the method of Sibat and Hasegawa [6] and the concentration of sodium hydrogen phosphate according to Fiske and Subbarow [7] using an electrophotocolorimeter FEK-1. The results of the determinations of phenol and sodium hydrogen phosphate from single samples usually agreed well with each other.

In numerous repetitions of the analyses of the studied solutions, especially when there was slight hydrolysis of the phenylphosphate, the results of the determination of phenol were the more constant. Therefore in most experiments sodium hydrogen phosphate was not determined and the rate of enzymatic hydrolysis of phenylphos-

phate was estimated only by the change in concentration of the phenol. It should be noted that the method of determination of phenol according to Sibata and Hasegawa is considerably simpler to carry out and is no less accurate than the generally known method of King.

In order to calculate the value of the spontaneous decomposition of phenylphosphate we always ran control experiments parallel with the main experiments, in which the phenylphosphate was hydrolyzed under the same conditions as in the chief experiments but in the absence of enzyme.

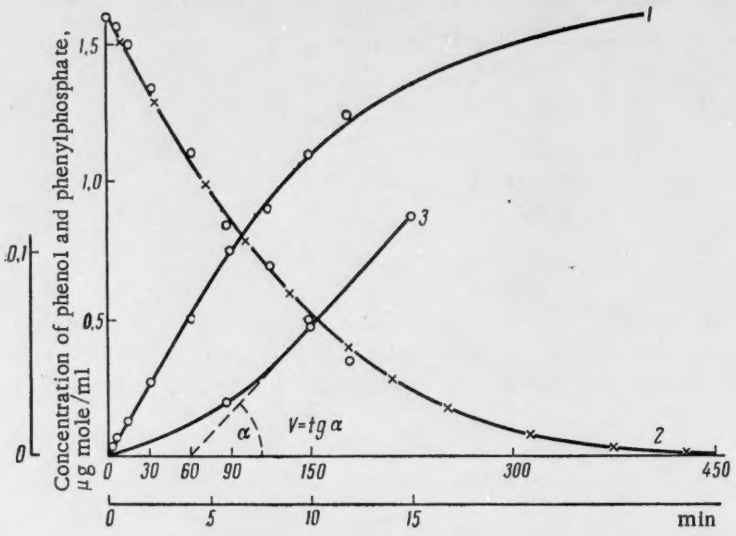

Fig. 1. Change in concentrations of phenol and sodium phenylphosphate during the reaction. Enzyme concentration 0.001 mg/ml; 1) change in phenol concentration; 2) change in phenylphosphate concentration; 3) change in phenol concentration (large scale). Circles, experimental data; crosses, calculated by equation (16).

The value of the phenol concentration C_p was determined in the reaction mixture at different intervals of time $\underline{t}$ and is plotted on the graph in coordinates $C_p - \underline{t}$ (Fig. 1, curve 1). From the experimental curves we calculated the initial reaction rate $\underline{V}$.

At small enzyme concentrations, as a rule during the first two minutes after mixing the solutions of enzyme and substrate, there were practically no hydrolysis products in the reaction mixture; there was a latent period in the enzyme reaction (Fig. 1, curve 3). In this connection, for the initial reaction rate we conditionally used a value corresponding to the moment when the reaction was stabilized; in this case, the moment corresponding to a time of 7 min (Fig. 1, tangent). Strictly speaking the initial reaction rate is zero.

The concentration of undecomposed phenylphosphate in the reaction mixture C_s is calculated by the equation $C_s = C_{s,0} - C_{pS}$ where $C_{s,0}$ is the initial concentration of substrate in the reaction mixture. Using this formula we constructed the curve in the coordinates $C_s - \underline{t}$ from the observed values of phenol concentration (Fig. 1, curve 2).

Study of enzymatic hydrolysis of phenylphosphate in vessels of different shapes. Some authors [8, 9] have considered that many enzyme reactions occur by a chain mechanism. The existence of a maximum rate of enzymatic hydrolysis of phenylphosphate in the coordinates $V - C_s$ gives a basis for speaking of the possibility of a chain mechanism in the present reaction. In this connection it is important in setting up the experiments to show the effect of shape of the vessel on rate of hydrolysis of phenylphosphate.

The reaction mixture with a volume of 10 ml was placed in vessels with different shapes: in a tube with diameter 20 mm, in a conical retort with diameter 60 mm, in a tube with diameter 20 mm into which was inser-

ted a glass tube with inner diameter 7 mm and wall thickness 1 mm. In all three vessels after an equal time interval we found practically the same amount of formation of phenol and sodium hydrogen phosphate. Hence the rate of enzymatic hydrolysis of phenylphosphate does not depend on the shape of the vessel.

Dependence of rate of enzymatic hydrolysis of phenylphosphate on concentration of enzyme and substrate. For the study of the relation of reaction rate to concentration of enzyme we ran three sets of experiments. Each set consisted of four experiments in which the concentration of substrate was the same and the concentration of enzyme was different. The results of determination of initial reaction rate in all three sets are shown in Table 1.

TABLE 1

Relation of Reaction Rate to Enzyme Concentration. Here and in Table 2 the reaction rate is expressed in μg mole/ml/min; the upper value was obtained experimentally, the lower was calculated by equation (15)

Set	Substrate concentration, μg mole/ml	Concentration of substrate, μg mole/ml			
		0,00025	0,0005	0,001	0,002
I	0,8	0,002 0,00145	0,00325 0,0029	0,0063 0,0058	0,0131 0,0116
II	2,5	0,0030 0,00264	0,0062 0,00528	0,012 0,0106	0,0245 0,0212
III	8	0,00238 0,00219	0,00438 0,00438	0,0085 0,00876	0,0187 0,0175

From these results it follows that the rate of enzymatic hydrolysis of phenylphosphate is directly proportional to the enzyme concentration.

For a study of the relation of reaction rate to substrate concentration we ran two sets of experiments. Each set consisted of seven experiments in which the concentration of enzyme was constant and the concentration of substrate was different. The results of the determination of initial reaction rate in both sets are given in Table 2.

TABLE 2

Relation of Reaction Rate to Concentration of Phenylphosphate

Set	Enzyme concentration, mg/ml	Concentration of substrate, μg mole/ml						
		1	2	3	4	5	8	12
I	0,002	0,0130 0,0134	0,020 0,019	0,022 0,0224	0,0235 0,0235	0,0230 0,0233	0,0157 0,0175	0,0088 0,0091
II	0,004	0,0265 0,0267	0,0405 0,0388	0,0425 0,0448	0,0466 0,0471	0,0466 0,0466	0,0346 0,0351	0,0188 0,0188

With increasing concentration of substrate the rate of enzymatic hydrolysis at first rose and then fell. In the experimental curve calculated from the data of Table 2 we find that the maximum rate occurs in the region of concentration of substrate from 4 to 4.5 μg mole/ml and its position under the conditions given does not depend on enzyme concentration.

Experiments with dilution of the reaction mixture. Many authors [4, 10] connect the presence of a maximum rate of an enzymatic reaction with the formation of an inactive intermediate complex of enzyme with substrate. This point of view is not without basis and actually is the only one which explains the seeming contradiction between the law of mass action and the observed relation of rate of enzymatic hydrolysis of phenylphosphate to its concentration in the reaction mixture.

According to the Le Chatelier principle, with increasing concentration of substrate there should be an increased amount of enzyme bound in the inactive complex, and a corresponding fall in active concentration of

enzyme. Actually on increasing the concentration above $C_{S\ max}$ (the concentration of substrate which corresponds to the maximum reaction rate), the reaction rate does not increase, but decreases. In describing the kinetics of the enzyme reaction it is important to know how stable this inactive complex is. In this connection we ran experiments with dilution of the reaction mixture.

In a tube we placed equal volumes of solution of phenylphosphate and enzyme, previously heated in a thermostat at 37°. In the resulting reaction mixture the concentration of phenylphosphate was 12 μg mole/ml, and the concentration of enzyme was 0.00033 mg/ml. After 60 min we pipeted 5 ml of reaction mixture from the tube and placed it in a second tube with 5 ml of diluted buffer solution. Along with this by mixing corresponding solutions of substrate and enzyme we prepared a third (control) reaction mixture which in composition scarcely differed from the second, that is, the concentration of phenylphosphate in it was 6 μg mole/ml and the concentration of enzyme was 0.000167 mg/ml. We determined by analysis the change in concentration of the phenol in all the reaction mixtures and then calculated the values for the reaction rates.

The results of one of the typical experiments with dilution of the reaction mixture was as follows:

Reaction mixture	Enzyme concentration, mg/ml	Phenylphosphate concentration, μg mole/ml	Reaction rate in μg mole/ml/min	
			experimental	calculated
Concentrated	0.00033	12	0.00158	0.00419
Dilute	0.000167	6	0.0023	0.00181
Control	0.000167	6	0.0022	0.00181

It was shown that after dilution of the reaction mixture the rate of enzymatic hydrolysis of phenylphosphate was not decreased, but on the contrary, increased, in spite of the twofold decrease in concentration of substrate and concentration of enzyme. This can only be explained by assuming that dilution increases the active concentration of the enzyme because of partial decomposition of the inactive complex.

The agreement of the reaction rates in the second and third reaction mixtures gives reason to believe that the rate of formation of the inactive complex when the solutions of substrate and enzyme are mixed (third reaction mixture) is of the same order as the rate of splitting of the inactive complex in dilution of the concentrated reaction mixture (second reaction mixture). The results of these experiments show conclusively that the reaction of formation of the inactive complex is reversible and that equilibrium in this reversible reaction occurs relatively rapidly.

THEORETICAL PART

Derivation of the kinetic equation. In deriving the kinetic equation for the hydrolysis of phenylphosphate we have analyzed the various schemes for the enzymatic reaction. The most adequate of these is that which is based on the assumption of formation of two intermediate complexes of the enzyme F with the substrate S: an active complex FS and an inactive complex FS_{n+1}. We assume that the inactive complex is a product of the reaction of the active complex FS with n molecules of substrate. These molecules, bound to the active complex by the action of residual valence forces of the enzyme hinder the reaction of the active complex with water. Our mechanism has the form:

$$F + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} FS + H_2O \xrightarrow{k_3} F + P_1 + P_2,$$
$$FS + nS \quad (k_3 \downarrow\uparrow k_4) \quad FS_{n+1},$$

where P_1 and P_2 are hydrolysis products, in our case phenol and sodium hydrogen phosphate. We write the equations for the reaction rates of this scheme:

$$V_1 = -\left(\frac{dC_S}{dt}\right)_1 = \left(\frac{dC_{FS}}{dt}\right)_1 = k_1 C_F C_S; \tag{1}$$

$$V_2 = \left(\frac{dC_S}{dt}\right)_2 = -\left(\frac{dC_{FS}}{dt}\right)_2 = k_2 C_{FS}; \tag{2}$$

$$V_3 = \left(\frac{dC_{FS_{n+1}}}{dt}\right)_3 = -\left(\frac{dC_{FS}}{dt}\right)_3 = k_3 C_{FS} C_S^n; \tag{3}$$

$$V_4 = -\left(\frac{dC_{FS_{n+1}}}{dt}\right)_4 = \left(\frac{dC_{FS}}{dt}\right)_4 = k_4 C_{FS_{n+1}}; \tag{4}$$

$$V_5 = \frac{dC_p}{dt} = -\left(\frac{dC_{FS}}{dt}\right)_5 = k_5 C_{FS}, \tag{5}$$

where k is the rate constant for the corresponding reaction, C the concentration of the compound indicated by the subletter. The total decrease in concentration of the substrate dC_S/dt is expressed by the difference $V_1 — V_2$ which is denoted by $V_{1,2}$.

$$\frac{dC_S}{dt} = V_{1,2} = V_1 - V_2 = k_1 C_F C_S - k_2 C_{FS} = k_1 (C_{F_0} - C_{FS} - C_{FS_{n+1}}) C_S - k_2 C_{FS}, \tag{6}$$

where C_{F_0} is the initial concentration of enzyme which is the sum of the concentration of free enzyme F and the concentration of the enzyme bound in the intermediate complexes FS and FS_{n+1}.

The increase in concentration of inactive complex $dC_{FS_{n+1}}/dt$ is determined by the difference $V_3 - V_4 = V_{3,4}$.

$$\frac{dC_{FS_{n+1}}}{dt} = V_{3,4} = V_3 - V_4 = k_3 C_{FS} \cdot C_S^n - k_4 \cdot C_{FS_{n+1}}, \tag{7}$$

whence

$$C_{FS_{n+1}} = \frac{k_3 C_{FS} \cdot C_S^n - V_{3,4}}{k_4} = K \cdot C_{FS} \cdot C_S^n - \frac{V_{3,4}}{k_4},$$

where K is the equilibrium constant for the reversible reaction of formation of the inactive complex. Establishing the value of $C_{FS_{n+1}}$ from equation (7), in equation (6) we get:

$$V_{1,2} = k_1 \left(C_{F_0} - C_{FS} - K C_{FS} \cdot C_S^n + \frac{V_{3,4}}{k_4}\right) C_S - k_2 C_{FS},$$

whence

$$C_{FS} = \frac{k_1 C_{F_0} C_S + \frac{V_{3,4}}{k_4} - V_{1,2}}{k_2 + k_1 C_S + K C_S^{n+1}}.$$

The value of C_{FS} is established in equation (5)

$$V_5 = k_5 C_{FS} = \frac{k_5 \left(k_1 C_{F_0} C_S + \frac{V_{3,4}}{k_4} - V_{1,2}\right)}{k_2 + k_1 C_S + K C_S^{n+1}}. \tag{8}$$

At first the rate of formation of the products of enzymatic hydrolysis of phenylphosphate is zero, since at $t = 0$, $C_{FS} = 0$. At this moment $V_{3,4} = 0$ and $V_{1,2} = k_1 \cdot C_{F_0} C_S$ and therefore the numerator on the right hand side of equation (8) equals zero. In the course of time $V_{1,2}$ decreases and $V_{3,4}$ first rises and then falls. Thus a stationary state is gradually established in the system which is characterized by the fact that the reversible reaction of formation of the inactive complex approaches equilibrium and $V_{3,4}$ tends toward zero, the rate of increase in concentration of the products of hydrolysis V_5 becomes equal to the rate of decrease in concentration of substrate $V_{1,2}$, that is, the number of molecules of substrate reacting chemically equal the number of molecules of reaction product formed in the same time.

Then, with the occurrence of the stationary state, instead of equation (8) we obtain:

$$V = \frac{k_5 (k_1 C_{F_0} C_S - V)}{k_2 + k_1 C_S + K C_S^{n+1}}, \text{ whence } V = \frac{k_5 \frac{k_1}{k_2 + k_5} \cdot C_{F_0} \cdot C_S}{1 + \frac{k_1}{k_2 + k_5} C_S + \frac{k_1}{k_2 + k_5} K C_S^{n+1}}.$$

We introduce the new symbols:

$$\frac{k_1}{k_2 + k_5} = a; \quad \frac{k_1}{k_2 + k_5} K = b; \quad k_5 \frac{k_1 C_{F_0}}{k_2 + k_5} = r C_{F_0} = f;$$
$$C_S = C; \quad \text{and then}$$
$$V = \frac{r C_{F_0} C}{1 + aC + bC^{n+1}} = \frac{fC}{1 + aC + bC^{n+1}}. \tag{9}$$

From these equations we draw two conclusions which agree completely with the experimental results: 1) the rate of hydrolysis of phenylphosphate at a given concentration in the reaction mixture is directly proportional to the enzyme concentration; 2) at a given enzyme concentration in the reaction mixture the rate of hydrolysis of phenylphosphate at first rises with increasing concentration of substrate, and then, after reaching a maximum, falls with further increase in concentration of substrate.

At the maximum point $dV/dC = 0$. Differentiating equation (9) with respect to C:

$$\frac{dV}{dC} = \frac{f(1 - bnC_{max}^{n+1})}{1 + aC_{max} + bC_{max}^{n+1}} = 0, \text{ whence } b = \frac{1}{nC_{max}^{n+1}}. \tag{10}$$

Hence the value for the concentration of substrate at which there is a maximum rate of hydrolysis of phenylphosphate depends on the value of the equilibrium constant K for the reversible reaction $FS + nS \rightleftarrows FS_{n+1}$ and on the value of n, but does not depend on the concentration of enzyme in the reaction mixture, which also agrees with the experimental results.

Integrating equation (9) we get the equation

$$ft = \ln \frac{C_0}{C} + a(C_0 - C) + \frac{b}{n+1}(C_0^{n+1} - C^{n+1}), \tag{11}$$

which expresses the change in concentration of substrate with increase in time in which the enzymatic reaction takes place.

If the inactive component is not formed, then $K = 0$ and hence also $b = 0$ and then equation (4) is changed into the generally known Michaelis-Menten equation:

$$V = \frac{r C_{F_0} C}{1 + aC},$$

and equation (11) becomes:

$$ft = \ln \frac{C_0}{C} + a(C_0 - C). \tag{13}$$

Finding the constants a, b, r, and n. From equation (9) we get

$$\frac{1}{C} + bC^n = \frac{f}{V} - a. \tag{14}$$

In the coordinates $1/C + bC^n - 1/V$ equation (14) gives a straight line. This line intersects a segment on the ordinate equal to a with a negative sign, and the tangent of the angle formed by the line with the abscissa equals f. For the construction of such a line, besides the experimental data for V and C we must know n and b. We arbitrarily take for the value of n the whole numbers 1, 2, 3, and 4. For each value of n by equation (10) we calculate the corresponding value of b from the experimentally established values of $C_{max} = 4.3$ μg mole/ml:

$$b_1 = \frac{1}{C^2_{max}} = \frac{1}{(4,3)^2} = 0,05401;$$

$$b_2 = \frac{1}{2C^3_{max}} = \frac{1}{2\cdot(4,3)^3} = 0,00629;$$

$$b_3 = \frac{1}{3C^4_{max}} = \frac{1}{3\cdot(4,3)^4} = 0,000975;$$

$$b_4 = \frac{1}{4C^5_{max}} = \frac{1}{4\cdot(4,3)^5} = 0,00017.$$

Hence, equation (14) takes the form $(1/C) + 0.054C = (f/V) - a$ for n = 1; $(1/C) + 0.0063C^2 = (f/V) - a$ for n = 2; $(1/C) + 0.000975C^3 = (f/V) - a$ for n = 3; $(1/3) + 0.00017C^4 = (f/v) - a$ for n = 4.

We establish the values of V and C in this equation, obtained by graphical use of the experimental data (Table 2, series II). The results of the calculation are given in Table 3 and graphically present in Fig. 2.

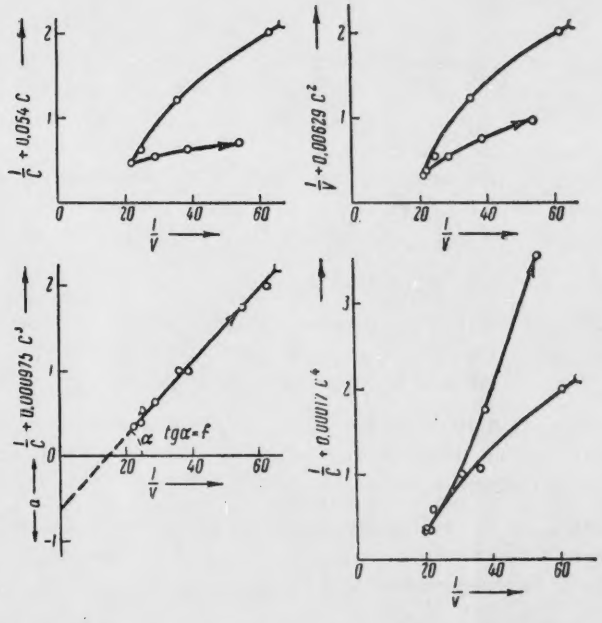

Fig. 2. Diagram of $1/C + bC^n - 1/V$ for the constants found in the kinetic equation for enzymatic hydrolysis of sodium phenylphosphate.

Fig. 2 at once shows that our experimental data in this system of coordinates gives a straight line only at $\underline{n}=3$. On this straight line we find that $\underline{a}=0.6$, $\underline{f}=0.0043$, whence $\underline{r}=f/C_{F_0}=0.043/0.004=10.75$ where 0.004 is the concentration of enzyme in the reaction mixture in mg/ml. Thus, for the hydrolysis of phenylphosphate under the influence of our alkaline phosphatase we obtain the following kinetic equations:

$$V=\frac{10{,}75C_{F_0}C}{1+0.6C+0{,}000975C^4} \tag{15}$$

$$t=\frac{2{,}3\lg\frac{C_0}{C}+0{,}6\,(C_0-C)+\frac{0{,}000975}{4}\,(C_0^4-C^4)}{10{,}75C_{F_0}} \tag{16}$$

Evidently our method can be used to derive kinetic equations not only for enzymatic hydrolysis of phenylphosphate, but also for any other enzymatic reaction in which there is hindrance of rate of reaction by the substrate. Perhaps it can be used also to derive kinetic equations for enzymatic reactions in the presence of reversibly acting inactivators.

TABLE 3

Data for Finding Constants in the Kinetic Equations of Enzymatic Hydrolysis of Phenylphosphate

C, μg mole/ml	v, μg mole/ml/min (from experimental curve)	$\frac{1}{C}$	$\frac{1}{v}$	$\frac{1}{C}+0{,}054\cdot C$	$\frac{1}{C}+0{,}0063\cdot C^2$	$\frac{1}{C}+0{,}000975\cdot C^3$	$\frac{1}{C}+0{,}00017\cdot C^4$
0,5	0,016	2	62,5	2,027	2,002	2,000	2,000
1	0,028	1	35,71	1,054	1,006	1,001	1,001
2	0,041	0,5	24,39	0,608	0,525	0,508	0,503
4	0,0465	0,25	21,50	0,466	0,351	0,312	0,293
5	0,0464	0,20	21,55	0,470	0,357	0,322	0,306
6	0,0436	0,167	22,94	0,491	0,394	0,378	0,387
7	0,0345	0,125	28,98	0,557	0,527	0,624	0,821
10	0,026	0,100	38,46	0,640	0,729	1,075	1,800
12	0,0184	0,083	54,35	0,731	0,989	1,768	3,608

Testing the derived equations. As the results show the values for the rate of enzymatic hydrolysis of phenylphosphate given in Table 2, calculated by equation (15) agree well with the experimental findings. In Table 1 (first set of experiments) and in the experimental data for dilution of the reaction mixture the calculated values are below the experimental ones. In these experiments we used another enzyme solution and therefore the differences are connected either with some inhomogeneity in our enzyme preparation, or with an error in weighing out its 10 mg. The values determined in the experiments for decreasing concentration of phenylphosphate with increasing time of proceeding of the reaction agree well with those calculated by equation (16) (Fig. 1).

SUMMARY

As a result of an experimental investigation we have established that the rate of enzymatic hydrolysis of phenylphosphate is directly proportional to the enzyme concentration. With increasing concentration of substrate the rate of hydrolysis at first rises and then falls, as was found by other authors.

We have derived kinetic equations for the hydrolysis of phenylphosphate under the influence of alkaline phosphatase, starting from the assumption of formation of an active complex FS and an inactive complex FS_{n+1} and have worked out a method for finding the constants in these equations.

We have found by mathematical treatment of the experimental results that the composition of the inactive complex should correspond to the formula FS_4. The value for the rate of reaction calculated by the derived equation agrees well with the value found experimentally.

By integrating the basic kinetic equation we have obtained an equation which expresses the change in concentration of substrate with increasing time of running the reaction, which also agrees well with the experimental results.

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THE DISTRIBUTION OF CHLOLINESTERASES IN THE BRAIN OF MACACUS RHESUS MONKEYS

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There are only limited data in the literature on the detailed quantitative topography of enzymes, and especially of different forms of cholinesterases, in the brains of animals and humans. In most of the work carried out by chemical methods there are data on total activity of cholinesterases in separated brains [1-3].

Burgan and Chipman [4] studied cholinesterase activity in ill areas of the cerebral cortex and also a number of nuclei and conducting paths of the brain and spinal cord of dogs. The sharpest difference was noted in the content of acetylcholinesterase in the cerebral cortex (about 200 units) and caudate nucleus (somewhat less than 4000 units). Benet and co-workers [5] succeeded in showing a statistically significant difference in cholinesterase activity in the cortex and subcortex of brains of rats of different genetic lines, and also a relation of cholinestearse activity in brain tissue to age of the animal.

The major role which is assigned to the acetylcholine-chloinestearase system in synaptic transmission justifies carrying out further detailed studies of the distribution of this important enzyme in parts of the brain which carry out different physiological functions. In the present communication we report materials on the study of the topography of cholinestearase in the brain of macacus rhesus monkeys.

METHODS

The investigation was carried out on macacus rhesus monkeys obtained through the Zoological Center from China. The weight of the animals varied from 3 to 6 kg. The animals were kept in cages and maintained on the standard ration.

Determination of the enzyme was carried out by the method of differential determination of the activity of acetyl and butyryl cholinesterases [6]. This technique, which relies chiefly on the principie of drop analysis, permits a quantitative study of the enzymatic process with an accuracy of 5-10%, using altogether several milligrams and also an amount of a milligram of studied tissue.

The principle of the method consists in determining the time required for splitting a definite percent of the choline ester used in the reaction: butyryl-choline in determining the nonspecific esterase and acetylcholine for the sum of the activities of the specific and nonspecific cholinesterases. The reaction time is established by the rate of change in color of bromthymol blue indicator occurring in a standard buffer solution due to liberation of acid in the hydrolysis of the ester. The reaction takes place in a colorimeter for drop reactions in the cup of which is placed a microdrop of a water extract from the tissue and the indicator-buffer solution of substrate. The supplying of the components is carried out with a specially calibrated dropper. The animals were killed by decapitation, the brain was quickly removed from the skull and placed in ice. From symmetrical portions of the cerebral cortex, cerebellum, subcortex samples were taken and their weight was determined on a torsion balance. The selected portions of brain substance with weight from 5 to 50 mg were carefully ground in a mortar with 50 or 100 times by weight of distilled water. The resulting suspension was transfered to a tube and allowed to stand over

night in a refrigerator; the settled out, homogeneous, slightly opalescent tissue extract (if necessary after supplementary centrifuging) was used for the further analysis.

Two drops of this extract were placed in the cup of the colorimeter with a calibrated pipet. For each extract we used four cups; two served for colorimetric standards corresponding to the initial and final fixed color of the reaction mixture, and two were used for determining the rate of hydrolysis of acetyl and butyrylcholine (the setting up of the first colorimetric standard was not essential). In the first cup we placed two drops of the main working indicator-buffer solution,* in the second, two drops of the same solution to which was added HCl in a concentration of 0.00125 M, in the third, two drops of a solution to which was added acetylcholine in concentration of 0.005 M, and in the fourth, two drops of main solution to which was added butyrylcholine in concentration of 0.005 M. Thus, the color of the contents of the second cup corresponded to the color of the reaction mixture which occurred after splitting of 25% of the ester used for the reaction. The contents of the cups were stirred with a fine glass rod.

In the third and fourth cups under the influence of the enzymatic splitting of the ester there was liberated acid which lowered the pH and produced a change in color of the indicator from blue to yellow. We measured the time for equalization of the color of each of the samples studied with the green color of the second standard.

Calculation of the enzymatic activity was carried out by the formula $A = 0.63\ V/T$, where T is the time of equalization of the color of the sample studied with standard No. 2 in minutes; V is the relation between the weight of tissue taken for study and the final volume of the reaction mixture; 0.63 is the amount of acid in μmole liberated in hydrolysis of 25% of the choline ester used in the reaction per ml of reaction mixture; A is the activity of cholineesterase expressed as micromoles of choline ester split in 1 g of tissue in 1 minute.

This formula permits direct calculation on the basis of time of hydrolysis of butyrylcholine of the value for the activity of the nonspecific butyrylcholinesterase, since this ester is hydrolyzed very slowly by acetylcholinesterase. The time of hydrolysis of acetylcholine cannot be determined on the basis of exact calculation of the activity of acetylcholinesterase, since this compound is hydrolyzed with considerable speed not only by acetylcholinesterase, but also by butyrylcholinesterase. Correction for the hydrolysis of acetylcholine by butyrylcholinesterase can be calculated by the formula

$$A_{(ac)} = A_{(z)} - 0.5A_{(bc)},$$

where $A_{(ac)}$ is the activity of acetylcholinesterase ; $A_{(z)}$ is the sum of the activities of acetyl- and butyrylcholinesterases in the material studied with respect to acetylcholine; $A_{(bc)}$ is the activity of butyrylcholinesterase calculated in the time of hydrolysis of butyrylcholine; 0.5 is the coefficient of recalculation (see [6]). The value of this correction for the cerebral cortex is from 10-15% of the total cholinesterase activity of the tissue studied; for the nuclei of the extrapyramidal system it is much lower, and for the conducting paths it is slightly higher.

TABLE 1

Extraction of Acetylcholinesterase by Water from Brain Tissues. Activity expressed in μmole/min/g

Tissue	1-day extraction	2-day extraction	Suspension of insoluble residue of tissue	30 min extraction				Suspension of insoluble residue of tissue	Enzyme in first extraction liquid %
				I	II	III	IV		
Cortex	2.2	0.05	<0.05	2.4	0.2	0.1	0.05	0.0	88.8
Nucleus caudatus	50	0.81	<0.05	63	7.8	0.87	0.1	0.0	87.5
Area olfacteria	63	1.26	<0.05	63	4.6	0.42	0.1	0.0	92.6

*The technique of preparing the solution is given in the work of Pokrovskii [6]. An exception is solution No. 2 in which we used half the hydrochloric acid called for in the work mentioned.

We first ran experiments to study complete extraction of cholinesterase from brain tissue of cortex, caudate necleus, and area olfactoria was ground by the usual method with 50-100 fold excess of water. On the next day the precipitate was separated by centrifuging and again ground with water. At the end we obtained a suspension of the insoluble part of the tissue in water. The two extracts and the suspension were studied by the usual method. Analogous experiments were run with extraction of the same tissue each 30 min. The results are given in Table 1.

Table 1 shows that this method of treatment with one day extraction gives a 96-98% extraction of the enzyme.

RESULTS

We studied the activty of butyryl- and acetylcholinesterase in 30-50 symmetrical parts of the cerebral cortex of the monkey brains.

We studied both the regions of the neocortex and the oldest parts of the cortex, especially the olefactory area with the tractus olfactorius and the olfactory bulb. We also studied in considerable detail the nuclei of the extrapyramidal system which carry out the higher unconditioned reflex activity of the organism, including the more recent formations, (caudate body and putamen) and the older ones (globus pallidus). We studied separately the activity of the conducting paths of the corpus callosum, the white matter of the hemispheres, optical tracts, etc. Of the diencephalon formations we studied the enzymatic activity of the optic thalamus nucleus and also the region of the hypothalamus which carries out the functions of the higher vegetative centers, including the tuber cinereum, mastoid body, which is related to perception of olfactory stimuli, hypophesis, etc. Of the mesencephalon formations we studied the anterior node of the lamina quadrigemina, related to perception of light stimulus, and the posterior node, connected with perception of auditory stimuli, and also the substantia nigra sommeringii which evidently coordinates acts of mastication and deglutition. Of the rhombencephalon formations, the same study was carried out on the bottom of the rhomboid fossa and especially the ala cinerea which is related to localization of the centers of the vagus nerve and also the nuclei of the pons varolii, superior olive of the medulla oblongata, etc. In all we carried out 8-12 determinations of cholinesterase activity in 52 morphologically and functionally different formations of the monkey brain.

The data which show the distribution of cholinesterase activity by zones are given in Table 2. It is quite evident that the activity of acetylcholinesterase in the cerebral cortex is about 4-7 times higher than is the activity of the butyrylcholinesterase. There is no great difference observed in the activity of the different parts of the cerebral cortex; it is somewhat higher for zones of motor analyzers as compared to sensory analyzers and in zones of the frontal pole and Gyrus supramarginalis it is somewhat lower than in the other parts of the cortex, but the differences are not great. The activity of the parts of the cortex at the base of the brain actually do not differ from the activity of the other parts, for instance those localized around the gyrus centralis. We note the extremely high activity in the olfactory area, which is about 50 times greater than the activity of acetylcholinestearase in the other zones of the cortex.

We also note the relatively high content of acetylcholinesterase in the olfactory bulb and even in the olfactory tract. In the nuclei of the thalamus and hypothalamus the content of acetylcholinesterase is also 5-6 times higher than the content of the enzyme in the cortex. It should be mentioned that the activity of butyrylcholinesterase in the portions with high acetylcholinesterase activity is increased to a considerably lower degree, so that the size of the coefficient which relates the activity of the two cholinesterases is very low, reaching a value of a few thousandths for the olfactory area instead of the usual 0.2-0.3 for the cortex. In most of the conducting paths the content of acetylcholinesterase is rather low, which results in a high value for the coefficient K.

We note the high content of cholinesterase in the nuclei of the extrapyramidal system. Actually, in the different portions of the corpus caudatus the activity of the acetylcholinesterase varies from 38.9 to 46.8 μmoles. Also very high is the content of acetylcholinesterase in the putamen, while in the globus pallidus the activity of the enzyme approaches the corresponding values for the nucleus of the thalamus. The enzymatic activity is relatively high in the formation of the midbrain; here the anterior tuber of the lamina quadrigemina in all cases contains considerably higher concentration of the enzyme than does the posterior tuber. Analysis of the acetylcholinesterase content of the rhombencephalon establishes the relatively high content of acetylcholinesterase over the whole extent of the bottom of the rhomoboid fossa. Similar figures characterize the activity of the gray matter of the cortex of the vermis and hemisphere in the cerebellum.

The most interesting and newest of the results given here is the fact of the existence of a considerable difference in acetylcholinesterase activity between the phylogenetically older and the more recent parts of the cerebral cortex (about tenfold). It should be noted that we found a similar relation in studies of human and dog brains.

TABLE 2

Level of Cholinesterase Activity in Different Formations of the Normal Monkey Brain. Activity of enzyme expressed in μmole of substrate

Region studied	Acetyl-choline A(ac)	Butyryl-choline A(bc)	K = A(bc)/A(ac)	Region studied	Acetyl-choline A(ac)	Butyryl-choline A(bc)	K = A(bc)/A(ac)
Telencephalon				Hypothalamus	5.98	0.89	0.15
Anterior gyrus centralis				Mastoid bodies	3.85	0.52	0.14
Motor analyzer of the leg	1.45	0.38	0.26	Tuber cinereum	4.12	0.36	0.087
Motor analyzer of the hand	1.66	0.39	0.23	Hypophesis	0.62	0.37	0.6
				Conducting Paths			
Motor analyzer of the head and neck	1.12	0.32	0.28	Corpus callosum	0.48	0.51	1.1
				Commissura anterior alba	0.36	0.31	0.86
Posterior gyrus centralis				Optic chiasm	0.50	0.26	0.52
Cutaneous analyzer of the leg	1.12	0.32	0.28	Tractus opticus	0.48	0.27	0.57
				Corpus striatum			
Cutaneous analyzer of the hand	1.10	0.30	0.27	Corpus caudatus, anterior nucleus	38,9	0.71	0.018
Cutaneous analyzer of the head and neck	1.11	0.25	0.22	Corpus caudatus, medial nucleus	46.8	0.74	0.016
Superior frontal gyrus	1.18	0.29	0.25	Corpus caudatus, lateral nucleus	45.2	0.72	0.016
Lateral frontal gyrus	1.26	0.38	0.30	Putamen	41.0	0.99	0.02
Frontal pole	1.05	0.25	0.24	Globus pallidus	6.73	0.67	0.10
Superior temporal gyrus	1.17	0.23	0.20	Diencephalon			
Central temporal gyrus	1.49	0.23	0.15	Substantia nigra Sommeringii	8.92	0.93	0.10
Superior temporal gyrus in depth of sulcus temporalis superior	1.50	0.29	0.193	Brain stem	6.3	0.73	0.12
				Nucleus pontis varolii	6.31	0.75	0.12
Gyrus supramarginalis	0.94	0.28	0.30	Lamina quadrigemina, anterior tuber	9.23	0.88	0.096
Zone around operculum occipitalis	1.33	0.29	0.22	Lamina quadrigemina, posterior tuber	5.14	0.72	0.14
Zone around fissura calcarina	0.96	0.23	0.24	Rhombencephalon			
Superior frontal gyrus	1.73	0.29	0.17	Rhomboid fossa			
Cortex above trigonum olfactorium	1.47	0.27	0.18	Alla cinerea	6.66	0.49	0.07
				Trigonum hypoglossus	5.29	0.77	0.11
Cuneus	1.10	0.23	0.21	Colliculus facialis	5.48	0.71	0.13
Rhinencephalon				Bottom of rhomboid fossa, middle	5.5	0.75	0.14
Olfactory bulb	4.75	0.45	0.09				
Olfactory tract	3.2	0.42	0.13	Bottom of rhomboid fossa, upper	7.0	0.82	0.12
Area olfactoria	6.8	0.47	0.007				
Mesencephalon				Olives of medulla oblongata	5.56	0.83	0.15
Optic thalamus, anterior nucleus	4.91	0.53	0.11	Cerebellum			
				Cortex of vermis, top	5.65	0.42	0.07
Optic thalamus, medial nucleus	6.40	0.7	0.11	Cortex of vermis, bottom	4.27	0.44	0.11
				Cortex of hemisphere	4.0	0.35	0.09
Optic thalamus, lateral nucleus	6.81	0.67	0.098	White matter with nuclei	2.31	0.46	0.2

We can assume that such a marked quantitative difference in level of enzyme activity of the tissues of the cerebral cortex must be related to a serious reorganization of the biochemical mechanisms which carry out the physiological functions of these parts of the nervous system organs.

SUMMARY

We have observed a marked quantitative inhomogeneity in distribution of acetylcholinesterase in the brain of Macacus rhesus monkeys. The highest activity of this enzyme was found in the region of the area olfactoria and the new nuclei of the extrapyramidal system: the lowest amount of the enzyme was found in the cerebral cortex. In the motor zone of the cortex the acetylcholinesterase content was somewhat higher than in the sensory zone.

When we compare the data which characterize the peculiarities of distribution of cholinesterases in the brain of humans, monkeys, and dogs we find a definite general plan of enzymatic constitution of the brain.

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GLUTAMINASE AND ASPARTASE IN GASTRIC MUCOSA

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While the connection of the functional state of the nervous and muscular systems with the processes of formation and removal of ammonia has been studied in detail in many cases at the present time, both here and abroad, the question raised by Pavlov and Nentskii [1] as to the connection of ammonia with the secretory function of the stomach still remains less studied. We have found in our work [2] that glutamine is present in the gastric mucosa and that its amount can be increased as a result of binding ammonia by glutamic acid. We suggested that the glutamine in the gastric mucosa, which is the form of bound ammonia in it, at the same time is one of the sources of ammonia formation.

In the present work we give data on the presence of the enzyme glutaminase in the gastric mucosa and its topography there.

METHODS

At the present time [3] two glutaminases have been detected in organs: glutaminase I which deaminates glutamine with formation of free ammonia and glutamic acid, and glutaminase II which along with the deamination carries out transfer of the amino groups to ketoacids. In this work we have studied only glutaminase I, activated by phosphate. The determination was carried out by the method of Mardashev and Lestrovaya [4] with slight modification.

After decapitation of the animals, which had been starved for one day, the mucous membrane of the stomach was carefully washed with water, separated off, and ground with liquid air. A homogenate was prepared from the resulting mass in physiological saline in the ratio: 1 g of tissue to 9 ml of solution. Incubation was carried out in a Conway vessel at room temperature for 3 hours. The incubation mixture consisted of 2 ml of homogenate, 2 ml of 0.2 M phosphate buffer (pH 8), 2 ml of 0.01 M glutamine or asparagine.

In the inner chamber of the Conway vessel was put 2 ml of 0.01 N H_2SO_4. After incubation, 2 ml of saturated K_2CO_3 solution was added to the incubation mixture. After 24 hours we determined the ammonia by nesslerization using an FEK-M photoelectric colorimeter. The deaminase activity was expressed in micrograms of ammonia nitrogen ($N\text{-}NH_3$) from 1 g of tissue after 3 hours.

At the same time we ran two controls: one on glutamine or asparagine with water instead of homogenate, and the other on homogenate instead of amide solution. The amount of ammonia found in both controls was subtracted from the amount of ammonia found in the experimental incubation mixture. In a number of cases for comparison we ran parallel determinations of the glutaminase activity in the brain and kidney of the same animals.

RESULTS

The results of the determination of glutaminase activity in the gastric mucosa are given in Table 1.

After establishing the glutaminase present in the gastric mucosa, it was of interest to determine its activity in the different parts of the stomach which differ in functional secretory characteristics: in the large and small curvature and in the pyloric portion. The results of these experiments are given in Table 2.

TABLE 1

Glutaminase in the Gastric Mucosa. Activity in μg N-NH_3/g tissue after 3 hours

Experimental animal	Gastic mucosa	Brain	Kidney	Experimental animal	Gastric mucosa	Brain	Kidney
Cat	204	592	662	Cat	240	—	—
"	273	753	793	"	364	—	—
"	402	882	862	Kitten	352	—	—
"	509	847	549	"	522	—	—
"	236	—	—	Dog	434	—	—
"	263	—	—	"	274	—	—
"	302	—	—	"	434	—	606
"	573	—	—	Puppy	550	—	—
				"	566	—	—

TABLE 2

Glutaminase in the Gastric Mucosa of Different Parts of the Stomach. Activity in μg N-NH_3 /g tissue after 3 hours

Experimental animal	Small curvature	Experimental animal	Pyloric portion	Experimental animal	Large curvature	Small curvature	Pyloric portion
Cat	301	503	231	Cat	353	441	226
"	104	130	0	Dog	388	462	344
"	274	379	171				
"	113	203	0	Dog (15 min after injection			
"	168	260	157	of histamine)	566	612	460

In the determination os aspartase in the gastric mucosa we obtained the following results:

Experimental Animal	Activity in μg N-NH_3/g tissue after 3 hours
Cat	84
"	175
"	198
"	0
"	0
"	136
Dog	50
"	71
"	4
"	14

DISCUSSION

We have not found data on the glutaminase of gastric mucosa in the literature. In the work of Richterich, Goldstein, and Cohen [3] along with other data there are also results on the glutaminase of the guinea pig stomach. However, the determination was carried out for the whole mass of the organ as is shown by the method of preparing the homogenate described in the work [3]. This is also indicated by the value for the glutaminase activity of the stomach reported in the work. On the average, it is about 15 times weaker than in the brain and 4 times weaker than in the kidney, two organs with the highest activity of glutaminase as compared to the other organs studied in the work.

The findings in the gastric mucosa of cats and dogs which we obtained are much higher. Since the activity of glutaminase in cats and dogs is of the same order, the average of all the determinations, 382 μg N-NH_3/g tissue after 3 hours, is about half the glutaminase activity of the brain and kidney. In other words, the secretory apparatus of the stomach approaches more closely than any other organ in this respect to the brain and kidney, in which glutaminase is the most important factor in ammonia formation. This means that in the gastric mucosa there is a very active glutaminase under the action of which ammonia is formed from glutamine, that is, in the secretory apparatus of the stomach the system glutamine-glutamic acid is not only a biochemical mechanism for binding ammonia, but is a very active source of its formation.

As to the connection of ammonia formation with the secretory function of the stomach, and in particular with the formation of hydrochloric acid, the data which we have obtained on the topography of glutaminase in the gastric mucosa are indicative. Its greatest content is found in the small curvature of the stomach, the least in the pyloric portion. This agrees with the data from the laboratory of Bykov [5, 6] on the isolation from the small curvature of the most acid juice and of its leading role in the development of the secretory processes of the whole stomach. On the other hand, in the pyloric portion which secretes an alkaline juice, the glutaminase activity is lowest and sometimes is not seen at all.

The activty of aspartase is much lower. It is very variable and in some cases is entirely absent.

SUMMARY

The gastric mucosa have a relatively high glutaminase activity, in some cases approaching the glutaminase activity of the brain and kidney which have the highest content of this deaminating enzyme. The connection of the secretory function of the stomach is indicated by the nature of the topography of this enzyme in the gastric mucosa, with the highest activity in the small curvature, which is the most active secretory potion of the stomach.

The activity of aspartase in the gastric mucosa is much lower and is less constant.

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ENZYMATIC OXIDATION OF ADRENALINE' NORADRENALINE AND TYRAMINE IN THE LIVER AND HEART OF RABBITS

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The analogy between the physiological activities of adrenaline, noradrenaline, and some other sympathomimetic amines consists in their corresponding, more or less, to the excitation effect of adrenergic nerves. This is why the different phenylalkylamine derivatives are included in the group of adrenergic substances. Many investigators consider the differences in the activities of adrenergic amines as due only to their being different in chemical structure, and do not consider the role of the metabolism of adrenergic substances in the mechanism of their action.

At the present time, many facts have become known in physiology and biochemistry, which show that in the study of the character and duration of the action of sympathomimetic amines, it is necessary to take into account not only their chemical structure, but also the rate and direction of their reactions [1-3].

The reactions of adrenergic substances in the organism comprise various paths of enzymatic and non-enzymatic oxidation, stabilization related to reaction with organic and inorganic compounds, inclusion in proteins, and liberation in effector organs, etc.

The majority of investigators consider that the enzymatic oxidation of noradrenaline and adrenaline in the organism may follow two pathways corresponding to the amine oxidase and quinoid mechanisms [4-6].

The amine oxidase oxidation is catalyzed by the enzyme,monoamine oxidase. Under the action of monoamine oxidase, adrenaline loses methylamine, homoprotocatechuic aldehyde being formed. If tyramine or noradrenaline are oxidized, the products are ammonia, and the corresponding aldehyde. As these products are physiologically inactive in the amounts which can be formed in the organism by the deamination of adrenaline, noradrenaline or tyramine, the amine oxidase reaction of sympathomimetic amines is considered to constitute one of the rapid mechanisms for their inactivation.

The oxidation of adrenaline by the quinoid mechanism is accomplished by metal-containing enzymes (polyphenol oxidase, cytochrome oxidase, etc.), and leads to the formation of a series of reversibly oxidized substances and quinoid structures, which are utilized in the metabolic processes as catalysts [7]. According to Utevskii, the products of quinoid oxidation of adrenaline lie in the reaction pathway of a hormone to a biocatalyst. However, it is not known at the present time what relative amounts of adrenaline and noradrenaline are oxidized in the organism either by the amine oxidase mechanism, or the quinoid mechanism.

The participation of monoamine oxidase in the oxidation of adrenergic amines is confirmed by a study of the distribution of this enzyme in the organism. Numerous investigations have shown that monoamine oxidase is found in tissues rich in amines and possessing an adrenergic innervation, such as the liver, the kidneys, the heart, the aorta, etc. [8-9]. Monoamine oxidase has not been found in animals deprived of chromatin cells. However, these facts do not constitute an indication that monoamine oxidase will oxidize in the same degree tyramine, noradrenaline and adrenaline, especially as these amines differ from one another in their physiological functions,

and distribution in the organism. Moreover, the same substrate is oxidized by the monoamine oxidase of different organs at different rates. Thus, the monoamine oxidase of the lungs, kidneys, and spleen oxidizes more rapidly 5-hydroxytryptamine, while the monoamine oxidase of the liver oxidized tyramine and tryptamine at a higher rate [10]. No convincing data on the oxidation of adrenaline by monoamine oxidase have appeared in the literature. Blaschko et al. [11] have shown that when adrenaline is incubated with liver monoamine oxidase, oxygen absorption diminishes upon addition of enzyme inhibitors. The authors concluded from this observation that adrenaline is metabolized by oxidative deamination. Indirect evidence in favor of the participation of monoamine oxidase in the breakdown of adrenaline has been obtained in experiments with inhibitors of the enzyme. Philpot [12] discovered that cocaine, procaine, and other local anesthetics inhibit the oxidation of adrenaline by 60-90%. However, the concentrations of anesthetics used by Philpot were too high as compared with the physiological. Schayer and other [13, 14] have found that introduction of labeled adrenaline in white rats led to the excretion with urine of substances related by their properties to the products of the reaction of adrenaline with amine oxidase. If the animals are first treated with Marsilid (inhibitor of amine oxidase) by subcutaneous injection, and then, with labeled adrenaline, the products of amine oxidase reaction of adrenaline are not detected in urine anymore. These data are given by the authors as evidence in favor of the existence of the amine oxidase pathway for the metabolism of adrenaline in the organism.

Recently, it was advanced that the necessary condition for the action of monoamine oxidase on adrenaline is the preliminary methylation of the ortho-hydroxyl group of the latter with the help of the enzyme ortho-transmethylase [15].

In the present work, the oxidation of adrenaline, noradrenaline and tyramine with the participation of the monoamine oxidase of rabbit liver and heart have been investigated. The oxidation of these amines with the participation of other enzyme systems, including these oxidizing adrenaline along the quinoid pathway has also been studied.

METHOD

For the determination of monoamine oxidase activity by the manometric method, Greasey [16] has proposed a procedure in which metal containing enzymes are inhibited by the addition of cyanide to the system, monoamine oxidase being insensitive to cyanide. To exclude the further oxidation of the aldehyde formed, it was proposed to inhibit the aldehyde oxidase with semicarbazide. Greasey determined that under these experimental conditions, each oxygen atom consumed corresponds to one molecule of oxidized substrate. As this method has been elaborated on the basis of experiments carried out with amine oxidase from liver mitochondria, and with tyramine as substrate, one cannot be sure that with other sources of monoamine oxidase (and, especially when working with homogenates and using adrenaline and noradrenaline as substrates) the method would work satisfactorily. For this reason, along with the determination of the oxygen consumed, we assayed the amount of ammonia and methylamine in experimental runs in which homogenates were incubated with the corresponding substrates and inhibitors.

Rabbits weighing 2.0-2.5 kg were used in our studies. The animals were decapitated, and their livers and hearts were rapidly removed. The organs were washed with cooled physiological solution. A 1.0 g aliquot of tissue was homogenized with 10 ml of cooled Sörensen phosphate buffer at pH 7.17, using a Potter-Elvehjem homogenizer. The degree of disruption of the cells was controlled under a microscope, and the homogenates were filtered through thick silk cloth to remove scraps of tissue.

One ml of a 10% liver or heart homogenate was treated in an incubation vessel with 0.2 ml of 0.1 M semicarbazide, 0.2 ml of 0.01 M KCN, and 0.2 ml of a 0.5% solution of substrate (tyramine, noradrenaline, or adrenaline). The mixture was made up to 2 ml with Sörensen phosphate buffer at pH 7.17. The same system, but without the substrate was used for the determination of residual respiration. The monoamine oxidase activity was calculated on the basis of the oxygen consumed in the presence of the corresponding substrate, with subtraction of the residual respiration. The participation of other enzyme systems in the oxidation of adrenaline, noradrenaline, and tyramine was studied by incubating them with the homogenates in the absence of inhibitors. At the same time the spontaneous respiration of the homogenates was determined, and after subtraction of its value, the oxygen consumed in the oxidation of the substrate was obtained. The formation of red products of quinoid oxidation of adrenaline in the vessels was followed in order to check the occurence of this oxidative process. In order to prevent the formation of melanins as a result of autoxidation of the products of quinoid adrenaline oxidation, semicarbazide

was added to the system with the effect that oxidation was stopped at the stage of adenochrome formation. According to amount of oxygen consumed by reaction mixtures containing semicarbazide, but free of cyanide, one could assess the activity of the enzyme systems oxidizing adrenaline along the quinoid path.

The vessels were saturated for 2 min with O_2, and the samples were then incubated in a Warburg apparatus at 38°. After a 10 min time for equalization of temperature, the amount of O_2 consumed was read every 15 min for 1 hr. After completion of incubation, protein was precipitated with trichloracetic acid (2% w.r. to final concentration), centrifuged off, and washed. The combined supernatant liquid and washings were assayed for ammonia and methylamine by Conway's method, with the subsequent titration of the residual sulfuric acid.

RESULTS OF THE STUDY

Incubation of adrenaline, noradrenaline and tyramine in a Warburg apparatus with liver homogenates, in the presence and in the absence of inhibitors was accompanied by an increased oxygen consumption, as compared with blanks in which the homogenates were incubated only with cyanide and semicarbazide. Data on oxygen consumption by liver homogenates with addition of cyanide and semicarbazide, and in the presence or the absence of substrate are given in Table 1.

TABLE 1

Dependence of the Influence of Cyanide and Semicarbazide (on O_2 Consumption by Liver homogenates) on the Presence of Adrenaline, Noradrenaline and Tyramine. Oxygen consumption in μl/sample

Substrates	Incubation time, min			
	15	30	45	60
Adrenaline	18±1,57	25±1,7	30±3,36	32±2,42
Noradrenaline	20±1,32	27±1,93	32±1,77	35±1,98
Tyramine	25±1,29	35±2,08	40±1,06	45±1,88
Without substrate	10±0,31	15±0,18	18±1,63	20±1,57

From Table 1 one can see that oxygen consumption by liver homogenates in the presence of tyramine is somewhat higher than in the samples containing adrenaline or noradrenaline. Cyanide and semicarbazide do not inhibit the spontaneous respiration of the homogenates completely; the residual respiration increases somewhat toward the 60th min of incubation.

If one considers that the increase in oxygen consumption under these experimental conditions accounts only for the oxidation of the substrate by monoamine oxidase, then one may conclude that all the amines investigated are acted upon by monoamine oxidase, tyramine being the substance oxidized the most actively. However, ammonia or methylamine determination after incubation of homogenates with substrates has shown that if ammonia is detected in the sample containing tyramine or noradrenaline, methylamine is practically absent in samples of liver homogenate incubated with adrenaline. The activity of liver monoamine oxidase determined on the basis of O_2 consumption and ammonia or methylamine formation is represented in Fig. 1.

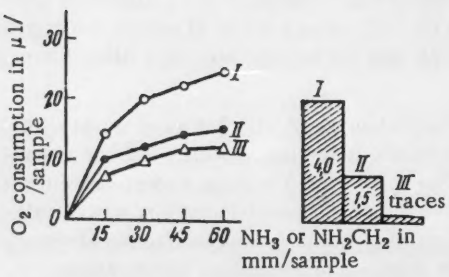

Fig. 1. Activity of liver monoamine oxidase calculated on the basis of oxygen consumption and ammonia and methylamine formation. I) Tyramine; II) noradrenaline; III) adrenaline.

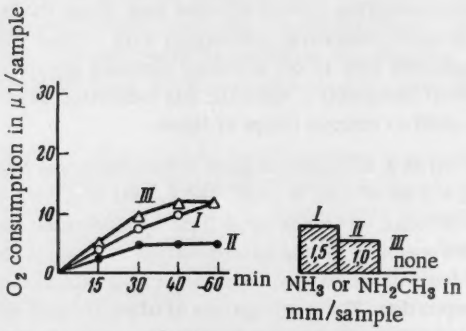

Fig. 2. Activity of heart monoamine oxidase calculated on the basis of oxygen consumption, and ammonia and methylamine formation. I) Tyramine; II) noradrenaline; III) adrenaline.

In Fig. 1, one observes a clear parallelism between oxygen consumption and ammonia formation in samples containing tyramine and noradrenaline: in samples with tyramine both oxygen consumption and ammonia formation were higher than the corresponding values with noradrenaline. On the other hand, only traces of methylamine were detected in samples with adrenaline as substrate, whereas oxygen consumption was almost at the same level as in samples with noradrenaline.

Experiments with heart homogenates showed that the highest oxygen consumption is observed when adrenaline is incubated in the presence of cyanide and semicarbazide. When noradrenaline and tyramine were incubated with heart homogenates in the presence of cyanide and semicarbazide, oxygen consumption was lower. Data on oxygen consumption by heart homogenates with addition of cyanide and semicarbazide, in the presence or absence of substrate are given in Table 2.

TABLE 2

Dependence of the influence of cyanide and semicarbazide (on O_2 consumption by heart homogenates) on the presence of adrenaline, noradrenaline and tyramine. Oxygen consumption in μl/sample

Substrates	Incubation time, min			
	15	30	45	60
Adrenaline	13±1,36	20±1,48	27±2,09	30±1,75
Noradrenaline	10±1,33	15±1,64	20±1,95	23±1,8
Tyramine	12±1,37	17±1,62	25±2,18	30±2,8
Without substrate	8±1,02	10±1,56	15±1,36	18±1,7

The determination of the activity of heart monoamine oxidase on the basis of O_2 consumption shows that this enzyme is active towards adrenaline, tyramine, and noradrenaline. Determination of ammonia or methylamine in the samples at the end of the incubation showed that heart monoamine oxidase is weakly active towards tyramine, and that it has a totally negligible activity towards noradrenaline. Under the given experimental conditions, it was not possible, in a single instance, to estimate methylamine. This allows us to conclude that monoamine oxidase does not deaminate adrenaline, the residual oxygen consumption in the case of cyanide and semicarbazide inhibition of respiratory enzymes being accounted for by other oxidative pathways. The activity of heart monoamine oxidase determined on the basis of O_2 consumption, and ammonia or methylamine formation is represented in Fig. 2.

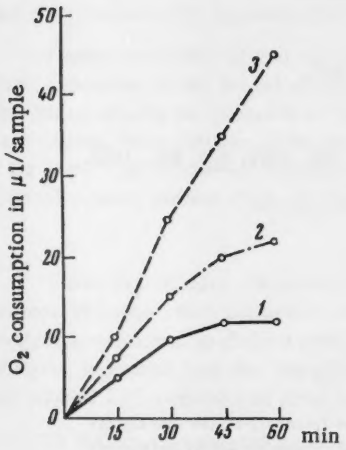

Fig. 3. Oxidation of adrenaline in the heart. 1) In the presence of cyanide and semicarbazide; 2) in the presence of semicarbazide; 3) without inhibitors.

By comparing the activity of liver and heart monoamine oxidase towards tyramine, one may conclude that this enzyme is more active in liver. This is in agreement with literature data. Noradrenaline is similarly deaminated in liver and heart, but less actively than tyramine. Under the given experimental conditions it was not possible to prove that adrenaline is deaminated either in liver, or heart. It is possible that preliminary reactions of adrenaline, such as methylation in ortho-position are necessary for its deamination by monoamine oxidase.

Data obtained during the study of the oxidation of adrenaline by heart homogenates without addition of cyanide or semicarbazide present a considerable interest. Incubation of adrenaline with heart homogenates in the absence of cyanide and semicarbazide leads to a high residual oxygen consumption in comparison with the spontaneous and residual respirations of homogenates, and to the appearance of red oxidation products of adrenaline. This is followed by autoxidation of quinoid structures with the formation of brown melanins. When the samples are free of cyanide, and contain semicarbazide, no conversion of red products into brown substances is observed, and the oxygen consumption after 45 min starts to lag somewhat behind the corresponding consumption by samples free of semicarbazide. Data obtained during the study of the oxidation of adrenaline in the heart are given in Fig. 3. During incubation of adrenaline with liver homogenates, the formation of red products is not observed.

Osinkaya's studies [17] have shown the presence in the heart of oxidation products of adrenaline having the properties of catechol-quinoid structures. By comparing these data with the results we obtained during the study of enzymic oxidation of adrenaline in the heart, one can conclude that the heart contains a metal enzyme system,

which oxidized adrenaline along the quinoid pathway; this is confirmed by the fact that no red oxidation products are formed in the presence of cyanide. It is possible that the quinoid pathway is characteristic for the heart, and that this type of oxidation does not occur in the liver. Further development of these data may serve for the elucidation of the preferential oxidation pathway of sympathomimetic amines in different organs.

SUMMARY

Oxidation of adrenaline, noradrenaline and tyramine by the liver and heart homogenates was followed up under the conditions of activity of monoamine oxidase and of metal containing enzymes.

It was shown that monoamine oxidase of the liver and heart deaminates tyramine and noradrenaline, the former more actively than the latter. Under the above experimental conditions no deamination of adrenaline was recorded.

Evidence was brought forward to show that the heart contains a metal enzymatic system which oxidizes adrenaline via the quinoid pathway which is not however the case with the liver.

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CHANGES IN THE ANTIGEN COMPLEXES OF BRUCELLA UNDER THE INFLUENCE OF PHAGE

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The previous work showed that the variant obtained under the influence of phage of one of the Brucella cultures differed in its chemical composition from the original strain. The purpose of the present communication is to study the antigen complexes of this variant.

As the material for the study we used culture variant 4 formed as a result of action of phage of the type abortus on the virulent culture Brucella abortus 146. The variant differed from the starting in change of morphlogy, loss of agglutinability, appearance of signs of dissociation and sharp weakening in virulency.

Further study showed that the chemical composition of the microorganisms of the variant had changed somewhat, depending on the period of keeping before growing the culture [2]. It was interesting to see whether these changes would also be reflected in the composition of the antigen complexes. For this purpose the antigen complexes were isolated from cultures of the variant grown directly after obtaining it, and from cultures grown after keeping it for 4 and 6 months at low temperature. For comparison, the antigen complexes were also extracted from the original virulent culture of Br. abortus 146.

METHODS

Three day cultures of Brucella grown on liver agar were washed with physiological saline and defatted with acetone and ether, then extracted with 5% TCA according to Boivin. After separation of the microbe mass the liquid was submitted to dialysis and lyophilic drying. The resulting preparation was dissolved in a small amount of water, separated from the insoluble portion by centrifuging, and again precipitated by acetone. The precipitate was washed with acetone and ether and finally dried in a vacuum desiccator.

In the dry antigen complex we determined the reducing substances according to Hagedorn-Jensen, nitrogen by Kjeldahl in the micromodification, phosphorus according to Fiske-Subbarow, uronic acids according to Dische, hexose amine according to Eson and Morgan, and RNA and DNA according to Schmidt and Tannhauser.

RESULTS

In the table we give yield and general chemical composition of the antigen complexes.

From a comparison of the results of analysis it is easy to see that the antigen complexes extracted by TCA from the variant differ markedly from those of the starting Brucella culture. The yield of variant antigen is about 1/3 the yield of antigen from Br. abortus 146. As compared to the antigenic substances of the starting culture, the variant antigen contains about three times as much reducing substance and 4.5 times more uronic acids. We also found a sharp decrease in content of hexose amine, DNA, phosphorus, and nitrogen. In distinction from the antigen of the starting culture, which contains only a slight amount of RNA, we observed in the variant antigen more than 2% RNA.

Chemical Characteristics of Antigen Complexes of Starting Culture and Variant 4

Cultures	Yield in % of microbe mass	Reducing substances	N	P	DNA	RNA	Hexose	Uronic acids
		in % of absolutely dry antigen						
Br. abortus 146								
Starting	4,55	16,48	2,84	1,12	3,58	0,09	5,28	1,54
Variant 4								
Grown directly after preparing	1,62	46,85	1,41	0,71	0,62	2,27	0,68	5,75
Grown after keeping 4 months	1,74	45,97	1,45	0,68	0,65	1,82	0,74	7,10
Grown after keeping 6 months	1,82	47,62	1,38	0,65	0,68	1,66	0,71	7,03

In the antigens of the variant cultures grown after long periods of keeping, we noted small variation in content of uronic acids and RNA. In general the chemical composition of the antigens obtained from cultures grown after different periods of keeping the variant was rather constant.

We reported previously [3] that the antigen complex extracted from Brucella by TCA consisted of two antigens with different chemical natures: an antigen of the Boivin type (AB) and an antigen which contains DNA (AN). Later the presence of DNA was noted by Braun and co-workers [4,5] and the suggestion was made of the possible role of this complex which contained DNA in the processes which determine the specific antigens and possibly their immunogenic properties.

In connection with this, it was of interest to make a comparative study of the differences of antigen complexes in the starting culture and the variants for their separate components.

For this purpose the antigen complexes were submitted to mild conditions of hydrolysis (0.1 N CH_3COOH for 4 hours on a boiling water bath). Under these conditions the antigen substances extracted from Brucella by TCA were separated into the antigen which contained nucleic acid and the separate components of the Boivin type antigen (polysaccharides, lipids, proteins) [3]. The resulting protein precipitate was separated by centrifuging and the clear hydrolyzate, after extraction of the lipids was fractionated by successive precipitations by acetone in the cold.

After addition of three times the volume of acetone the antigen complex which contained nucleic acid precipitated. It was separated and dried with acetone, ether, and in a vacuum desiccator. To the remaining liquid, acetone was added to ten times the volume and the fraction of free polysaccharides which came down was detached from the Boivin type antigen. In the AN antigen we determined the amount of nucleic acid, polysaccharide, and protein.

Fig. 1 gives the content of the different antigens and the components formed from them in the starting culture and variants. The antigen complexes extracted from the culture variants and also from the starting culture consisted of two antigens with different chemical natures. However, in the case of the variant antigen, the content of polysaccharide components was considerably higher. In the antigen of the Boivin type (Fig. 1) the polysaccharide fraction consisted of 80 against 40%, and in the antigen which contained nucleic acid, 75 against 45%. At the same time the variant antigen differed sharply in lower content of protein substances. Also, in the variant cultures there was a tendency to lowered content of antigen which contained nucleic acid.

Long keeping of the variant before growing the culture was not actually reflected in the composition of the antigen complexes. Some variation in content of separate components was found in the work with microbe cultures grown at different times.

Besides the quantitative measurement if the changes in content of the separate components in the variant antigen complexes, the latter could also be distinguished qualitatively from the corresponding components of the starting culture antigens. To settle this question, we studied the carbohydrate and amino acid composition of the proteins by paper chromatography.

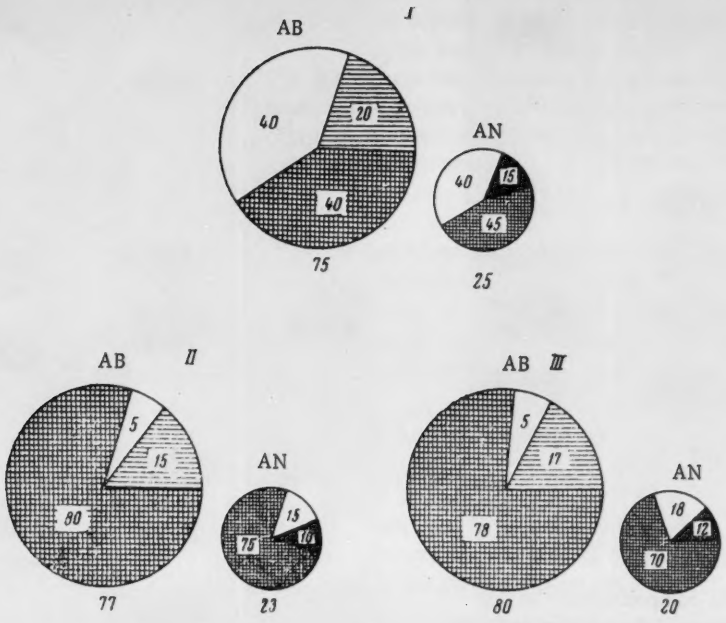

Fig. 1. Composition of antigen complexes extracted from Br. abortus 146 and variant 4 by trichloroacetic acid. I) Antigen of culture of Br. abortus 146; II) antigen of culture variant 4 grown directly after its production; III) antigen of culture variant 4 grown after 6 months keeping; AB, antigen of Boivin type; AN antigen containing nucleic acid. White areas, protein; striped area, lipid, cross-hatched area, polysaccharide; black area, nucleic acid.

First the polysaccharides were hydrolyzed with 1 N H_2SO_4 (4 hours on a water bath), and the protein with 6 N HCl (24 hours boiling). The hydrolyzate was freed from acids (in the case of proteins, from humin) desalted, concentrated, and deposited on paper in an amount corresponding to 600 μg of reducing sugars and 80 μg of protein.

Separation was carried out on Leningrad slow filter paper, using as the solvent butanol-pyridine-water-benzene (5:3:3:1) and butanol-acetic acid-water (4:1:5).

The chromatograms (Fig. 2) showed that the composition of polysaccharides of the variant antigen complex was more complicated than in the starting strain. They contained, in addition to uronic acid, galactose, and glucose, which form the specific polysaccharide of Br. abortus 146, also arabinose, xylose, and in lesser degree, ribose and rhamnose.

The specific polysaccharide of the variant culture grown after keeping for 6 months did not contain rhamnose. Evidently under conditions of long standing at low temperature this sugar is utilized in metabolism earlier than the other carbohydrate components.

The results of chromatographic analysis showed that the carbohydrate composition of both antigens of the variant culture actually differed from the corresponding carbohydrates of the antigen of the starting strain (Figs. 3 and 4).

The polysaccharide of the Boivin type antigen from Br. abortus consisted in the main of glucose and uronic acid. Only with deposition of an amount of hydrolyzate corresponding to more than 600 μg of reducing substances could we detect traces of ribose in it. In variant 4 the polysaccharide of the anitgen from the Boivin type consisted (except for arabinose) of the same monosaccharides as were found in the unseparated antigen complex of this culture. Just as in the unseparated antigen complex, in the Boivin type antigen from the culture variant grown after keeping for a time, there was no rhamnose. In the polysaccharide of the antigen complexes which contained

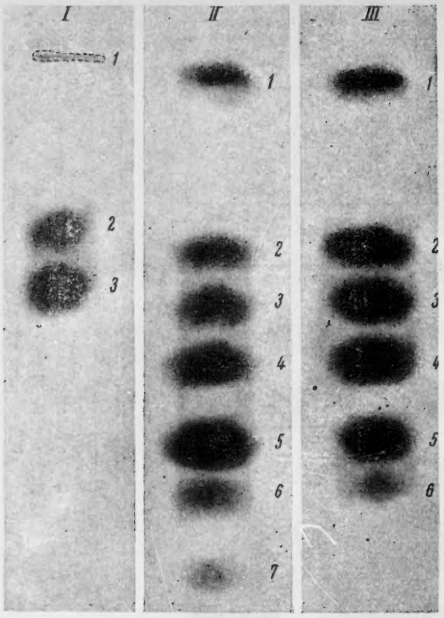

Fig. 2. Carbohydrate composition of the polysaccharide components of the unseparated antigen complex. Unsaturated antigen complex from: I) Culture of Br. abortus 146; II) culture from variant 4, grown immediately after obtaining; III) culture from variant 4 grown after keeping 6 months; 1) uronic acid; 2) galactose; 3) glucose; 4) arabinose; 5) xylose; 6) ribose; 7) rhamnose.

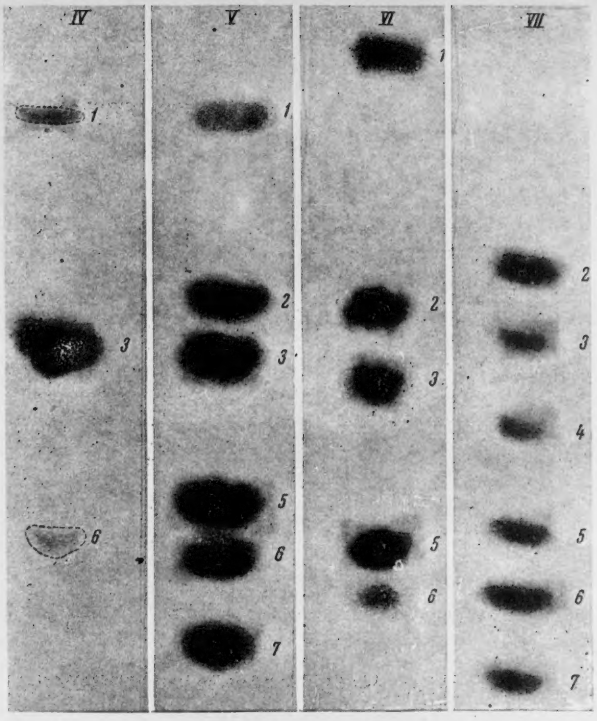

Figure 3. Carbohydrate composition of polysaccharides from Boivin type antigen. Polysaccharide of Boivin type antigen isolated from: IV) Culture of Br. abortus 146; V) culture of variant 4 grown immediately after obtaining; VI) culture of variant 4 grown after keeping 6 months; VII) standards. Other designations as in Fig. 2.

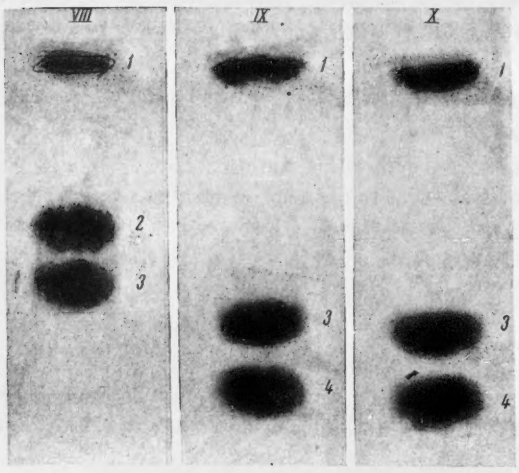

Fig. 4. Carbohydrate composition of polysaccharides of the antigen which contains nucleic acid. Antigen containing nucleic acid isolated from: VIII) Culture of Br. abortus 146; IX) culture of variant 4 grown immediately after obtaining; X) culture of variant 4 grown after keeping 6 months. Other designation as in Fig. 2.

nucleic acid we found arabinose in the variant instead of the usual galactose of the starting strain.

The increased content of the uronic acid in the polysaccharides of the variant cultures may perhaps depend on the intensive transformation of hexoses into pentoses, temporarilly fixed at the stage of uronic acid as one of the steps in the transformation. Thus the antigen complex of the variant differs from the antigen of the starting culture not only in the quantitative predominance of the polysaccharide components, but also in the qualitative selection of monosaccharides.

In view of the small content of protein, the study of its amino acid composition was carried out on the unseparated complex. For the chromatographic separation (Fig. 5) the amino acids of the hydrolyzate were separated on Leningrad slow filter paper and the solvents (successively) butanol-acetic acid-water (4:1:5) and butanol-acetic acid-water (40:15:5).

The protein composition of the variant culture antigens grown after keeping for various periods was the same. Differing from the proteins of the antigens

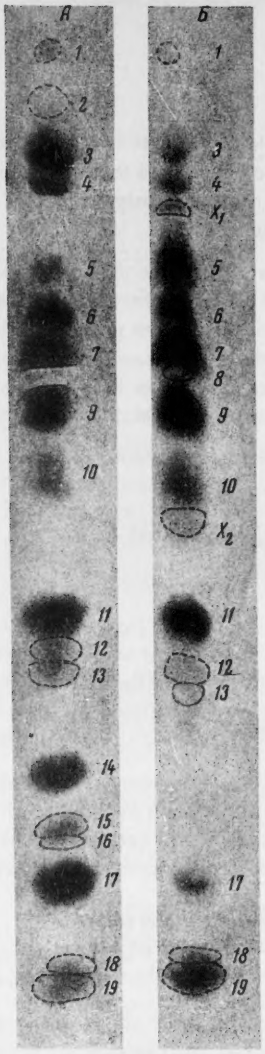

Fig. 5. Amino acid composition of proteins of antigens complexes from Br. abortus 146 and variant 4. A) hydrolyzate of proteins from antigen of Br. abortus 146; B) hydrolyzate of protein from antigen of variant 4. 1) Cystine; 2) lysine; 3) histidine; 4) arginine; 5) aspartic acid; 6) serine; 7) glycine; 8) hydroxyproline; 9) glutamic acid; 10) threonine; 11) alanine; 12) proline; 13) tyrosine; 14) α-aminobutyric acid; 15) methionine; 16) valine; 17) phenylalanine; 18) leucine; 19) isoleucine. X_1 and X_2 are unidentified amino acids.

of the starting culture, in the protein components of the variant antigen complex there were absent lysine, α-aminobutyric acid, methionine, and valine. Besides this, we noted in them hydroxyproline and two new unidentified amino acids. Under the conditions of this experiment, one of them (X_1) lay between arginine and aspartic acid, the other (X_2) between threonine and alanine.

DISCUSSION

Variant 4 was obtained along with other phage resistant variants from the virulent culture of Br. abortus 146 after action on it of bacteriophage of the type abortus [6]. Variant 4 differed from the starting strain by weakened virulency, and a change in morphological and specific properties.

In the previous study [1] we showed that the greatest chemical change which distinguished the cultures of the variant from the cultures of Br. abortus 146 was the shift in content of lipids, hexose amines, and composition of the nucleic acids, polysaccharides, and proteins. Such a change in content of protein and nucleic acids in animal or bacterial cells infected by virus or phage has been encountered in the studies of other authors [7, 8].

The results obtained in this work show that variant 4 differs markedly from the original culture in the composition of its antigen complex. It was further shown that not all the change in chemical, composition of the variant is of a stable character [2]. Variant cultures grown after prolonged keeping continued to differ from normal cultures of Br. abortus 146 chiefly in the composition of their antigen complex and hexose amine content. It was shown that the most stable chemical change occurred in the composition of the antigen complex and the most persistant of the newly arising properties of the variant is the weakening of the virulency. The question occurs, as to whether there is a connection between these effects.

One more circumstance should be considered. If we take into account that the antigen complex extracted from the microorganisms by the method of Boivin is distributed on the surface of the bacterial cell and is bound to the membrane, then it becomes evident that the surface layer on the variant cells has a different chemical composition than in the cells of the original Br. abortus 146.

In this connection we should note the work of Jesaitis and Göbel [9]. These investigators, studying the phage resistant variant of Shigella sonnei, also showed that it contained an antigen which differed in composition from the antigen of the original phage sensitive cells.

We can assume that in the phage resistant variants which have a different antigen complex qualitatively from that of the original culture there is replacement of one active group by another at the surface of the cells. This fact probably connected with the loss of a definite receptor which serves as a spontaneous protective mechanism for penetration of a specific phage into the micoorganism.

In the light of these results on study of the antigen complexes it is perfectly natural to find a change in serological properties in the phage resistant variant arising as a result of the action of phage on one or another of the

phage sensitive Brucella cultures.

SUMMARY

The weakly virulent variant 4 obtained from the virulent strain of Brucella abortus 146 by the action of phage contains markedly less antigen material extractable by trichloroacetic acid than does the original culture. The antigen of the variant differs from the antigen of Br. abortus 146 in higher content of polysaccharides and lower content of protein components, and also by a change in content of nucleic acids.

The polysaccharides and protein components of the antigens of the variant differ from the corresponding components of the antigen of the original culture. The specific polysaccharides of the variant are formed from a more complex selection of monosaccharides. In the amino acid composition of the antigen proteins of the variant there are absent α-aminobutyric acid, lysine, methionine, and valine, but there are present hydroxyproline and two unidentified amino acids. The antigen complexes of the variant cultures grown after keeping it for various periods, except for some changes in polysaccharide components are distinguished by considerable contancy in their composition.

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DETERMINATION OF THE AMINO ACID COMPOSITION OF POLYMYXIN M BY THE METHOD OF COUNTERCURRENT DISTRIBUTION

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In previous work [1] we have shown that the method of countercurrent distribution can be used for qualitative and quantitative determination of amino acids in mixtures by separating their 2,4-dinitrophenyl (DNP) derivatives. In the present communication we give results of the use of this method for determination of the amino acid composition of a new polymyxin (polymyxin M), the isolation and some of the properties of which were briefly described earlier [2-4].

Preliminary investigation [4] by paper chromatography and paper electrophoresis showed that in the composition of the polymyxin there occur threonine, α, γ -diaminobutyric acid, and amino acids of the leucine group. Therefore the first step in our work was to find the exact conditions of countercurrent distribution at which there would be complete separation of the DNP derivatives of these amino acids.

EXPERIMENTAL PART AND DISCUSSION OF RESULTS

As the result of a number of experiments it was established that a good separation of these DNP-amino acids was attained even after 100-110 transfers in the system: phosphate buffer, pH 7.55-butyl acetate.

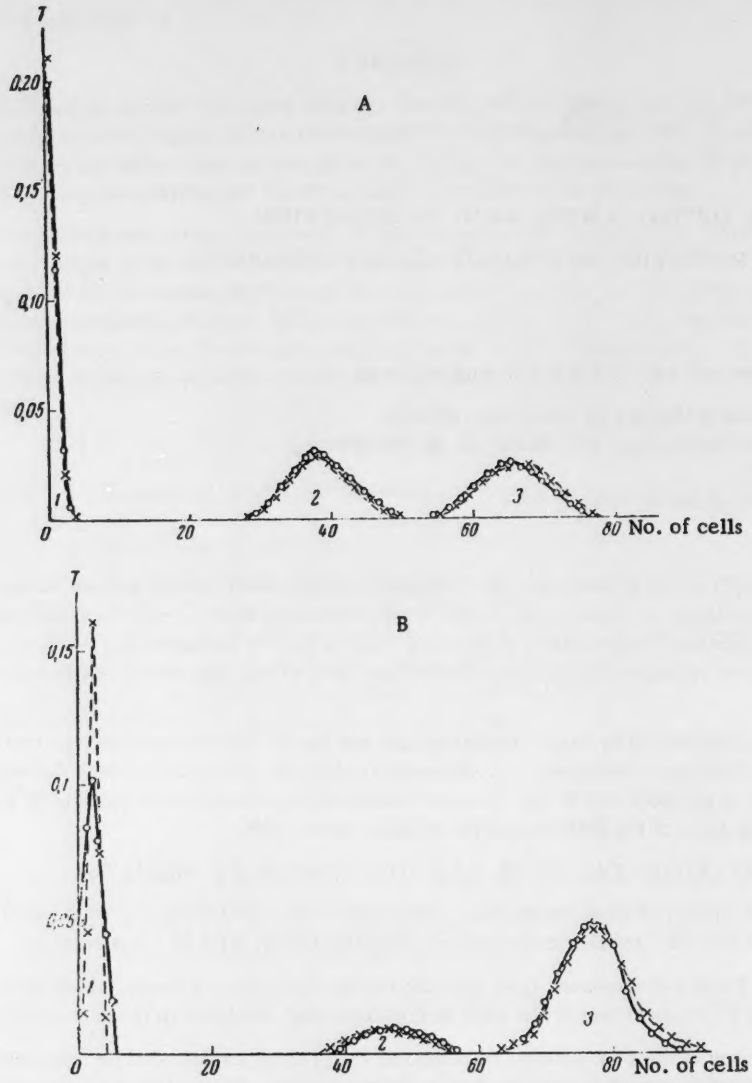
In Fig. A and in Table 1 (experiment 1) we give the results of these experiments, which show good agreement of the amounts of DNP-derivatives of the three amino acids used and found in the determination.

Successful separation of the DNP-derivatives permitted carrying out experiments on separation of mixtures of these amino acids. For this purpose it was necessary to determine the optimum length of the reaction of dinitrophenyl derivative formation. The experiments which were carried out showed that formation of the dinitrophenyl derivatives of the three acids with 2,4-dinitrochlorobenzene in 90% aqueous alcohol on the boiling water bath occurred at different rates, but in the course of eight hours we obtained good results for all the acids.

TABLE 1

Separation of Artificial Mixtures of 2,4-DNP-amino acids by Countercurrent Distribution

DNP-amino acid	109 transfers, pH 7.55						109 transfers, pH 7.4	
	Experiment 1			Experiment 2			Experiment 3	
	taken m-moles	found m-moles	%	taken m-moles	found, m-moles	%	found, mmoles	ratio
2,4-DNP-threonine	35,06	37,08	105	84,9	84,12	99	28,0	3,0
2,4-DNP-leucine	32,97	34,32	104	63,3	65,04	102	9,84	1,15
2,4-di(α,γ -DNP)-amino-butyric acid	29,3	30,3	103	44,49	44,1	99,1	60,0	6,2



Separation of artificial mixtures of 2,4-DNP-amino acids (A) and the hydrolyzate of polymyxin M in the form of the 2,4-DNP-derivatives of the amino acids (B). 1) 2,4-DNP-threonine; 2) 2,4-DNP-leucine; 3) 2,4-di (α, γ -DNP)-aminobutyric acid. T) amount of substance in each cell. Dotted line, theoretical curve; crosses experimental, curve.

In Table 1, (experiment 2) we give results of determining the quantitative ratio of the three amino acids by nitrophenyl derivative formation in eight hours and later separation by countercurrent distribution in the above phase system. The results show that in this experiment also we obtained good agreement of the taken and found ratio of amino acids.

After the preliminary study we ran experiments on determination of the amino acid composition of polymyxin M.

Forty mg of polymyxin M was hydrolyzed by heating on a boiling water bath with 18% H_2SO_4 in a stream of CO_2 for 30 hours. The resulting hydrolyzate was extracted with ether to remove fatty acids. For removal of

$SO_4^=$ ions the hydrolyzate was heated on a boiling water bath and repeatedly treated with small portions of $BaCO_3$ to pH about 6. The resulting $BaSO_4$ and excess $BaCO_3$ were filtered off, the precipitate was washed several times with small amounts of hot distilled water, and the hydrolyzate and wash water were evaporated in a vacuum to a volume of about 5 ml. In the resulting concentrated hydrolyzate we dissolved 40 mg (476 mmoles) of $NaHCO_3$ and then treated this solution with 60 ml of alcohol which contained 61 mg (302 mmoles) of 2,4-dinitrofluorobenzene. Reaction of formation of the dinitrophenyl derivatives was carried out for eight hours. Then the solution was acidified with acetic acid, filtered, and evaporated dry in a vacuum.

Half the resulting reaction product was separated in a countercurrent separation apparatus in the system: phosphate buffer, pH 7.4-butyl acetate.

Figure B shows that after 109 transfers the three substances were well separated from each other, and the experimental curve fully agreed with the theoretical curve. From the amount of the three substances found, shown in Table 1 (experiment 3) we can conclude that in polymyxin M there are three residues of threonine one residue of the amino acids of the leucine group, and six residues of α, γ-diaminobutyric acid.

In order to show the presence of these amino acids in polymyxin M, we had to isolate their 2,4-dinitrophenyl derivatives from the hydrolyzate of the antibiotic in pure form.

For this purpose we hydrolyzed 2 g of polymyxin M as described above. After treatment and dinitrophenyl formation in the hydrolyzate, the DNP-amino acids which formed were separated by countercurrent distribution. After 220 transfers we collected three fractions of substances from the tube near the maximum.

Treatment of the fraction which contained 2,4-DNP-threonine was carried out as follows. 2,4-DNP-threonine from butyl acetate solution was extracted several times with a 1/15 M solution of Na_2HPO_4; the combined extracts and the phosphate buffer solution were twice washed with ether to remove traces of butyl acetate and were acidified with 10% H_2SO_4 to an acid reaction to Congo. 2,4-DNP was extracted from the acidified solution with ethyl acetate. The ethyl acetate extract was dried with anhydrous Na_2SO_4 and evaporated in a vacuum at 40° to dryness. The residue was dissolved in 20 ml of ethyl ether and precipitated with petroleum ether (with b.p. not above 70°). The precipitate was recrystallized from a mixture of ether and petroleum ether. The resulting yellow crystals after drying in a vacuum up to 100° melted at 139-140°.

Formula of 2,4-DNP-threonine, $C_{10}H_{11}N_3O_7$. Calculated: C 42.1%; H 3.9%; N 14.5%. Found: C 42.22%; H 4.13%; N 14.25%.

By analogous treatment of the fraction which contained the DNP-derivative of α, γ-diaminobutyric acid we obtained a yellow substance with m.p. 142-145° which by analysis agreed well enough with the corresponding di-(dinitrophenylamino) butyric acid. Formula of the latter $C_{16}H_{14}N_6O_{10}$.

Found: C 42.7%; H 3.1%; N 18.7%; Calculated: C 41.85%; H 4.32%; N 18.12%.

In an analogous way we treated the fraction which contained the DNP-derivative of the amino acids of the leucine group. The substance isolated had m.p. 130-133°, which showed its possible identity with DNP-leucine. As is known, exact differentiation of isomers of leucine is a rather complex problem. In order to show rigidly that the isolated DNP-compound is actually a leucine derivative, and not a derivative of isoleucine or norleucine, we determined the content of C-CH_3 groups (according to Kuhn-Roth) in the isolated form and in known DNP-drivatives of leucine, isoleucine, and norleucine. We can assume that, as in other cases, the isopropyl group in leucine will give a small amount of acetic acid (about 0,5 mole/mole leucine). This assumption was confirmed and the results of the determinations (Table 2) left no doubt that polymyxin M contains leucine and not its isomers.

Thus we can consider that qualitatively and quantitatively the amino acid composition of polymyxin M has been exactly determined by countercurrent distribution of the dinitrophenyl derivatives of the amino acids which make up the antibiotic.

SUMMARY

By using countercurrent distribution of the dinitrophenyl derivatives of the aminoacids it is shown that polymyxin M contains one leucine residue, three threonine residues, and six α, γ-diaminobutyric acid residues.

We have worked out a process for exact differentiation of the amino acids of the leucine group, by the use of which we have shown that polymyxin M contains leucine and not its isomers.

TABLE 2

Determination of the Content of C-CH_3 Groups in DNP-Derivatives of Amino Acids

DNP-derivative	Structural formula	% CH_3	No. of C-CH_3 groups	
			theory	experimental
2,4-DNP-leucine	$(CH_3)_2CH-CH_2-CH(NH-C_6H_3(NO_2)_2)-CO_2H$	2,9	~0,5	0,57
2,4-DNP-norleucine	$CH_3-CH_2-CH_2-CH_2-CH(NH-C_6H_3(NO_2)_2)-CO_2H$	4,59	1	0,91
2,4-DNP-isoleucine	$CH_3-CH_2-CH(CH_3)-CH(NH-C_6H_3(NO_2)_2)-CO_2H$	8,88	2	1,76
2,4-derivative of amino acid from polymyxin M		2,72	—	0,53

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EXTRACTABLE NITROGENOUS COMPOUNDS OF FISH MUSCLE

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Our previous communication [1] dealt with some of the characteristics of the distribution of some non-protein nitrogenous compounds in the muscles of certain fishes. From that work, and from published works of other workers, it is clear that the composition of fish muscle with respect to those compounds differs from species to species. This difference is particularly pronounced for the dipeptides, anserine and carnosine, and for the amino acids histidine and β-alanine. The species investigated were divided into groups on the basis of the distribution of the four compounds mentioned. The muscles of the species in one group lacked the two peptides, but contained large amounts of either histidine or β-alanine. In other species carnosine or anserine was detected, together with one of the constituent amino acids of the peptide. Representative of yet another group contained neither the dipeptides, nor histidine or β-alanine, in their muscles.

The present investigation, which supplements that published earlier, was concerned with the further study of the distribution of soluble, non-protein nitrogenous compounds in fish muscle.

METHODS

The compounds assayed in the muscles were amino acids, the peptides, anserine and carnosine, creatine and creatinine, as well as residual nitrogen. Urea was also assayed in some specimens. The latter was assayed in Conway dishes, as ammonia liberated after complete decomposition with urease. All other experimental procedures have been described elsewhere [1]. All biological material was collected in the Black Sea region and in the Amur river basin. The following groups and specimens were investiaged: Of the Elasmobranchs, order Selachii – the dogfish (Acanthias acanthias), order Batoidea – the ray (Trygon pastinaka). Of the Teleostoi, the family Clupeidae – the Caspian herring (Clupea caspiolosa) and the spratt (Clupea sprattus), Salmonidae – the gorbusha (Oncorhynchus gorbuscha) and the Siberian summer salmon (O. keta). Of the Acanthopterygii – the bluefish (Pomatomus salatrix), the croaker (Corvina nigra), the wrosse (Crenilabrus tinca) and the gurnard (Trigla lucerna). Of the Pleuronectidae – the plaice (Pleuronectes flesus luscus); of the Apodes – the conger eel (Conger conger).

The data shown in the tables have been obtained with one representative of each species, and are expressed as mg% of fresh tissue. Water-free residue weight of muscle was determined on the majority of specimens.

RESULTS AND DISCUSSION

The first group investigated was that comprising the dogfish and the ray, whose muscles, like those of the skate investigated in the previous work, lack the dipeptides anserine and carnosine, but contain high amount of β-alanine. The amino acid composition of the free pool in the muscles of these species is also characterised by a high level of glycine and sarcosine. It can be seen from Table 1 that the qualitative compositon of the amino acid pool was found to be the same in two species. The differences in the quantitative distribution applied mainly to the basic amino acids arginine, lysine and ornithine, whose levels were higher in the dogfish than in the ray. It it known, from published data, that the muscles of Elasmobranchs contained high levels of urea. In the present work, the urea content of the ray muscle was found to be 475 mg%. In the teleostoi these levels were considerably lower.

TABLE 1

The Distribution of Some Nitrogenous Compounds in the Muscles of Elasmobranchs, Clupeidae and Acanthopterygii (in mg%)

Compound	Dogfish	Ray	Spratt	Herring	Bluefish
β-alanine	105	60	—	—	—
Histidine	—	—	250	160	250
Leucine + isoleucine	42	29	10	+	20
Phenylalanine	22	15	+	9	35
Valine	+	+	27	20	10
Tyrosine	—	—	24	15	7
Alanine	34	29	4	25	26
Sarcosine	99	81	—	—	—
Threonine	14	13	13	8	10
Glutamic acid	26	33	3	4	17
Glycine	50	60	6	13	12
Serine	21	16	4	15	+
Aspartic acid	15	12	7	10	
Arginine	50	7	+	6	+
Lysine	+	13	—	+	—
Ornithine	25	+	+	+	+
Urea	+	475	6	—	not determined
Creatine + creatinine	+	+	+	446	352
Residual nitrogen	1077	986	340	321	259
Dry tissue weight %	not determined	20,4	21,1	30,0	23,9

TABLE 2

Non-Protein Nitrogenous Compounds in the Muscles of Salmonidae (in mg%)

Compounds	Summer salmon		Gorbusha		
	III–IV	death	III	IV–V	death
Anserine	877	649	726	891	527
Histidine	14	18	7	8	6
β-Alanine	26	21	+	+	+
Methylhistidine	48	50	+	+	+
Leucine + isoleucine	26	27	4	5	4
Phenylalanine	21	10	+	3	+
Valine	3	10	4	17	0,4
Tryptophan	6	17	+	17	+
Tyrosine	12	16	2	8	traces
Alanine	25	21	14	5	2
Threonine	6	2	7	13	5
Glutamic acid	7	3	20	16	2
Glycine	14	13	40	32	5
Serine	9	+	21		4
Aspartic acid	12	+	26		1
Arginine	7	3	+	+	+
Lysine	25	17	+	+	+
Ornithine	+	+	+	+	+
Creatine and creatinine	175	200	424	472	520
Residual nitrogen	435	298	381	450	422

Note. Roman numerals denote various stages in the development of the sexual products.

The species of the second category investigated also lacked anserine and carnosine, but contained appreciable levels of histidine. The species were the spratt, the Caspian herring, and the bluefish. Histidine levels in the muscles of those fish were somewhat lower than in those of the species investigated previously, but were still higher than the levels of other amino acids in each species. The actual distribution of amino acids in the entire group (Table 1) shows a similarity of pattern in the various species. The quantitative differences in amino

TABLE 3

Non-Protein Nitrogenous Compounds in the Muscles of Acanthopterygii, Pleuronectidae and Apodes (in mg%)

Compound	Croaker	Wrosse	Cusk eel	Plaice	Conger eel
Leucine + isoleucine	+	23	15	9	53
Phenylalanine	+	19	20	16	54
Tyrosine	24	12	8	20	32
Proline	+	+	+	+	+
Alanine	15	42	19	26	74
Threonine	13	13	9	15	63
Glutamic acid	9	9	17	13	67
Glycine	18	28	15	15	57
Glycine	30	11	15	10	50
Serine				13	
Aspartic acid			13		
Arginine	4	+	10	12	81
Lysine	+	+	37	21	34
Ornithine	+	+	4	8	+
Cystine	+	+	+	+	+
Histidine	—	—	19	16	—
Creatine + creatinine	+	+	304	220	+
Residual nitrogen	239	280	300	253	489
Dry weight of muscle, %	21,8	20,0	19,0	20,3	19,6

acid content of the muscles are, apparently, a result of individual variations, which are quite pronounced in the various specimens.

The next group investigated consisted of the Salmonidae, whose muscles contained anserine and histidine. The salmon types investigated in the present work were the Far-Eastern species found in the Amur river basin—the gorbusha and the Siberian summer salmon. Muscles were sampled from fish in various stages of development of their sexual products and at the time of death. The spawning stage is known [2, 5] to bring about pronounced changes in the content of a number of compounds in various tissues of the salmon. At this period of life the muscles of the salmon carry an additional functional load. The increased muscular activity of fish whose feeding is completely abolished can be expected to cause considerable changes in the nitrogenous make-up of the muscle pool. Thus, considerable change in the amino acid composition of the muscles of the carp during the winter fast have been reported [3]. In the present work, no concomitant change in the amino acid composition of the salmon muscle with the development of the sexual products could be detected. However, depletion of the muscles of amino acids was observed before death, particularly severely in the gorbusha (Table 2). These changes were less pronounced in the muscles of the summer salmon. A characteristic property of the muscles of both species is their high anserine content. The presence of β-alanine and of methylhistidine was probably due to a partial cleavage of the peptide, whose content in the muscles declined towards the time of death.

The fish whose muscles completely lack anserine and carnosine, and virtually lack β-alanine and histidine, form a separate group. Here belong the fish of the perch family—the croaker, wrosse, bluefish and gurnard; the Pleuronectidae—the plaice; and the Apodes—the conger eel. As seen from Table 3, glycine and alanine predominate in the muscles of those fishes. In the muscles of all species in this group there is also another compound whose position, the chromatographic solvents employed, coincided with that of glutamine. It should be noted that, under the conditions of processing of the protein-free extract, glutamine was likely to be converted to glutamate or to another intermediate—pyrrolidone carboxylic acid—which does not react with ninhydrin. The unknown substance (possibly taurine), which overlapped with the glutamine spot, remained unidentified. Of the last group investigated, the conger eel is noteworthy because of the high content of all amino acids in its muscles. Quantitative determinations of amino acids were not carried out in the muscles of the gurnard. This species did not differ from the other fish of the same group in the qualitative composition of the muscle amino acid pool. Two, three or more specimens were studied in each species of this group, with the exception of the conger eel, of which species only one specimen was investigated.

The differences in the composition of the non-protein nitrogenous pools in various species of fish depend on a variety of factors related to their biological and ecological characteristics. However, the systematic classification of the given species also plays a significant role. This is demonstrated by the fact that the appearance

of carnosine and anserine in the muscles of the fish is characteristic for the more recent groups. Thus, one of the constituents of the dipeptides – β-alanine – occurs in the muscles of elasmobranchs, while culpeidae, the most ancient of the bony fishes, contain the other constituent amino acid – histidine. This phylogenetic difference is also known to be reproduced in the ontogenesis of single species. Thus in the early stages of the embryonal development of vertebrates β-alanine or histidine are absent, but in the later stages there appears carnosine, and later still, anserine.

SUMMARY

The two dipeptides, carnosine and anserine, were found to be lacking in the muscles of Elasmobranchia (Acanthias acanthias and Trygon pastinaka), but one of the constituent amino acids – β-alanine – was present. The muscles of those species contained, among other amino acids, large amounts of glycine and sarcosine.

The representatives of the Clupeidae examined (Clupea sprattus and Clupea caspialosa) and one of the Perciformes (Pomatomus saltatrix) also lacked the two dipeptides, but contained histidine.

The muscles of the Salmonidae of the Far East (Oncorhynchus keta and O. gorbuscha) contained large amounts of anserine.

The muscles of some Acanthopterygii examined (Corvina nigra, Crenilabrus tinca, Trigla lucerna, Ophidium barbatum) and those of one species each of Pleuronectidae (Pleuronectes flesus luscus) and Apodes (Conger conger) lacked both the dipeptides, as well as histidine and β-alanine. In the muscles of the entire group there are large amounts of an un-identified nitrogenous material, portion of which may be glutamine.

The author wishes to express thanks to Professor S. E. Severin for the suggestion of the topic of this work and continuous help in its progress.

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A QUANTITATIVE CHROMATOGRAPHIC ASSAY OF BILE ACIDS IN THE BILE AND BLOOD

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Conjugated bile acids predominate in the bile of healthy people and animals, and only a very small amount of bile acids occurs in the free state. In some liver diseases, the ability of this organ to synthesize conjugated bile acids is considerably perturbed with the result that the content of free bile acids in bile appreciably increases; this effect may have a definite diagnostic and prognostic importance. This stimulates an interest for the methods which allow fractionation of mixtures of bile acids, and assaying each of them separately. To serve this aim the chromatographic method for the separation of bile acids either on a column of various absorbents [2-4], or on chromatographic paper [5-7] has found the widest application. However, its drawback, which delays the wide use of paper chromatography in laboratory practice, is the rather long duration of the experiment, attaining 48 hours.

In the present work, a rapid and comparatively simple method for the chromatographic separation of bile acids on paper is described.

EXPERIMENTAL PART

Reagents used: Cholic acid obtained from KEK Company (m.p. after repeated recrystallization, 196 deg); desoxycholic acid of the same Firm (m.p. after repeated recrystallization, 176 deg); taurocholic acid, obtained from the bile of big cattle [1] (m.p., 185 deg); glycocholic acid, from the bile of big cattle [1] (m.p., 134 deg); chemically pure glacial acetic acid; chemically pure formic acid as a 85% solution; chemically pure toluene; chemically pure dichloroethane; twice distilled 96% ethanol; chemically pure 67% sulfuric acid; twice distilled methanol.

Chromatographic procedure: For the chromatographic separation of bile acids on paper, we used the ascending method, as it is the simplest chromatographic procedure available. Tests with different solvents as the mobile phase showed that best results are obtained with the following mixtures: Toluene–85% formic acid–glacial acetic acid–methanol (6 : 0.1 : 0.25 : 0.3) for cholic and desoxycholic acids, and dichloroethane–85% formic acid–glacial acetic acid–methanol (5 : 0.8 : 0.5 : 0.4) for taurocholic and glycocholic acids.

The chromatographic separation of bile acids was carried out as follows. Strips of chromatographic paper (2–2.5 x 28 cm) were used; on one end of the strips, 4.5 cm from the end, a horizontal line was drawn with a pencil. The alcoholic solution containing a mixture of bile acids (0.005 ml) was placed on this line with the help of a micropipette. A second line was drawn at a distance of 18.5 cm from the starting line. In a 3.5 x 30 cm glass cylinder was poured a freshly prepared solvent mixture (two cylinders were used: one for the solvent mixture suitable for free bile acids, and the other for the mixture suitable for conjugated acids). The cylinders were closed with rubber stoppers, and shaken to moisten their inner sides with the solvent mixture and thus speed up the saturation of the inner space with the vapors of the solvent. Strips of chromatographic paper with samples of bile acids were attached to the rubber stoppers with pins, and placed inside the cylinders in such a way as to have their free ends dipping in the solvent. The solvent front was allowed to raise to 18.5 cm above the starting line; the paper strips were then dried for 20 min at 80-90 deg. This was followed by spraying the strips with a

TABLE 1

R_f Values of Bile Acids for Different Kinds of Chromatographic Paper.

Chromatographic paper	t:f:a:m (6:0.1:0.25:0.3)		d:f:a:m (5:6.8:0.5:0.4)	
	R_f cholic	R_f desoxycholic	R_f taurocholic	R_f glycocholic
Leningrad, grade B	0,35	0,69	0,12	0,58
» grade 4-M	0,43	0,73	0,14	0,70
Whatman № 1	0,36	0,64	0,12	0,58
» № 4	0,40	0,64	0,14	0,64
» № 42	0,42	0,70	0,15	0,81
Schleicher and Schull № 595	0,59	0,79	0,17	0,75
» » № 597	0,50	0,67	0,25	0,81

Remark: t–toluene; f–formic acid (85%); a–glacial acetic acid; d–dichloroethane; m–methanol.

saturated solution of antimony trichloride in chloroform, and drying them at 90-95 deg for 5 min. The spots due to bile acids were located under ultraviolet light passing through a Wood's filter. The R_f values of bile acids determined with different kinds of chromatographic paper are given in Table 1. The duration of the chromatographic separation of bile acids by the foregoing procedure varies from one to three hours depending on the kind of paper used.

Chromatographic assay of bile acids in blood. Samples of blood plasma were preliminary subjected to a complementary treatment aiming at the removal of buffering substances which adversely affect the chromatographic separation and the reproducibility of results.

Two milliliters of plasma or blood serum was mixed with 8 ml of 96% alcohol, heated on a water-bath to boiling, and centrifuged from a precipitate which was washed with 2 ml of alcohol. The centrifuged washings were added to the main solution. Fatty acids, protein residues and pigments still contained in the centrifuged solution were precipitated by the addition of 1 ml of 0.25 N $Ba(OH)_2$ with subsequent heating for 1-2 min on a boiling water-bath. The precipitate was separated by centrifugation for 5-6 min at 6,000 r/min. The solution was then poured in a centrifuge tube, treated with 0.5 ml of 0.25 M $(NH_4)_2SO_4$ to precipitate excess of $Ba(OH)_2$, heated on a water-bath, and centrifuged for 3-5 min at 6,000 r/min. It is necessary to precipitate the excess of $Ba(OH)_2$, as otherwise, formation of $BaCO_3$ is liable to occur during the subsequent evaporation of the solution. This insoluble carbonate would then appear as a film sticking to the sides of the test tube and liable to retain bile acids. The centrifuged solution was decanted, and the residue washed with two portions of alcohol (1 ml each); the combined main solution and washings were evaporated under reduced pressure to a volume of 0.1–0.2 ml. The mixture containing the bile acids was then treated with 0.4 ml of 0.25 N $Ba(OH)_2$ and 2 ml of water and heated on a boiling water-bath. The precipitate of $BaSO_4$ which separated out was centrifuged out, and the solution decanted into a 20 ml separating funnel. The residue of $BaSO_4$ was washed with two 1.5 ml portions of distilled water with heating each time on a boiling water-bath. The combined main solution and washings were extracted three times with 3 ml of chemically pure ether saturated with distilled water. The lipids and cholesterol went into the etherial phase, which was separated from the aqueous-etherial phase. The latter was acidified with 0.05 ml of concentrated HCl, and the bile acids were then extracted with three 1.5 ml portions of n-butyl or isoamyl alcohol. The combined alcoholic extracts were evaporated on a water-bath under reduced pressure. If during the foregoing procedure the pigments were not completely precipitated, then they were removed with the help of a column of starch (the absorbent had to be thoroughly washed with water, alcohol, and ether, and dried at 50-60 deg for 24 hr). In order to carry this out, the residue left after evaporation of n-butyl or isoamyl alcohol was dissolved in 0.4 ml of ethanol and quantitatively placed on a starch column (9.5 x 0.8 cm). The column was washed with 3.5-4 ml of ether, which eluted the pigments. The bile acids, which remained on the column, were eluted with 3.5-4 ml of a 2:1 mixture of 96% ethanol and ether. The eluate was evaporated to dryness, and the residue dissolved in 0.1 ml of ethanol. The alcoholic solution was placed in 0.04 ml portions on two strips of chromatographic paper. One of the strips was dipped in the solvent mixture for free bile acids, and the other strip in the mixture for conjugated bile acids. Chromatography was carried out as described previously. The dried chromatograms were cut according to the position of the individual spots of bile acids, as in-

dicated by their Rf values, and the bile acids eluted with 96% alcohol between two slides as described by Sjövall [7]. The eluents were evaporated to dryness and the residues tested by Pettenkofer's reaction. Thus, the residue to be tested was treated with 0.4 ml of 1% fructose and 5 ml of 67% sulfuric acid. After adequate mixing, the mixture was heated for 15 min at 60 ± 1 deg. The cooled samples containing cholic, glycocholic, and taurocholic acids were assayed photometrically at 580 mμ against distilled water, and samples containing desoxycholic acid, at 520 mμ against a blank.

Assay of bile acids added to blood plasma gave the following results (in % w.r. to the added quantity):

Acid added mg%	Desoxycholic	Cholic	Taurocholic	Glycocholic
1	95.27 ± 1.32	97.34 ± 1.30	96.56 ± 1.22	98.98 ± 2.09
2.5	97.79 ± 0.81	98.10 ± 0.81	98.44 ± 0.80	98.90 ± 0.11
3.5	98.35 ± 0.82	99.57 ± 0.60	99.50 ± 0.60	99.00 ± 0.18

With the help of this method, we investigated the blood serum of ten healthy people and eight patients with mechanical jaundice. Data concerning the concentration of bile acids and bilirubin in the blood of patients with mechanical jaundice are given in Table 2.

TABLE 2

Concentration of Bile Acids and Bilirubin (mg%) in the Blood of Patients with Mechanical Jaundice

Diagnosis	Bilirubin in blood	Acids: Total bile	Glycocholic	Taurocholic	Cholic	Desoxycholic
Cancer of ampulla of Vater	2.58	3.0	1.4	0.7	Traces	–
Calculous cholecystitis and biliary cirrosis	12.00	7.9	2.73	1.47	2.68	Traces
Cancer of the general bile duct	10.85	2.5	1.6	0.8	Traces	–
Occlusion of the general bile duct	25.00	6.3	3.3	1.8	Traces	–
Cirrosis of the liver	3.75	2.0	1.2	0.55	–	–
Cancer of the pancreas	6.00	4.0	2.5	1.0	Traces	–
Cancer of the gall-bladder	10.40	2.0	1.44	Traces	1.0	–
Calculous cholecystitis	3.00	1.7	1.4	Traces	–	–

Bilirubin in blood was assayed by Vanderberg's method as modified by Malloya and Evelyn [8]. The total bile acids content was determined in 2 ml of plasma, after a preliminary purification in the same manner as before running paper chromatography. Pettenkofer's reaction was carried out on the residue obtained after purification. No bile acids were detected in the blood of healthy people.

Chromatographic assay of bile acids in bile. One tenth of a milliliter of filtered gall-bladder bile was treated with 0.9 ml of 96% ethanol; the mixture was heated for 30 sec on a boiling water-bath, cooled, and centrifuged. With the help of a micropipette, 0.012 ml portions of the centrifuged solution were placed on two strips of chromatographic paper (2 x 28 cm). Chromatography, treatment of the chromatograms, elution of bile acids, and quantitative assay were carried out in the way described for blood.

It is not necessary to purify bile before chromatography as the concentration of cholesterol, fatty acids and lipids in the samples used (0.012 ml of tenfold diluted bile) is very low and does not interfere with the results of the analysis. The bile pigments, which are present in the bile samples, do not influence the Rf values of bile acids, and either move on the chromatographic paper with the solvent front (when the solvent is the one used for conjugated acids), or remain on the starting line (during the chromatography of free bile acids).

Assay of bile acids added to bile gave the following results (in % w.r. to the added quantity):

Acid added mg%	Desoxycholic	Cholic	Taurocholic	Glycocholic
2	99.95 ± 0.57	100.58 ± 0.58	99.26 ± 0.08	100.80 ± 0.79
5	99.90 ± 0.12	100.75 ± 0.30	99.93 ± 0.10	100.20 ± 0.20

SUMMARY

New solvents for paper chromatography of bile acids, allowing carrying out a chromatographic assay in one to three hours, are proposed.

Chromatographic methods for the quantitative assay of bile acids in bile and blood are proposed. The accuracy of the method is 95.27 ± 1.32 to 99.57. ± 0.82% in the case of blood, and 99.26 ± 0.08 to 100.8 ± 0.79% in the case of bile.

Serum from ten healthy people and eight patients with mechanical jaundice was investigated by the proposed method. Bile acids were not detected in the blood of healthy people. The concentration of bile acids in the blood of patients with mechanical jaundice was 1.7 to 7.9 mg%.

No parallelism was found between the concentration of bilirubin and the concentration of bile acids in the blood samples from patients with mechanical jaundice.

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TRANSAMINATION OF DICARBOXYLIC AMINO ACIDS AND THEIR AMIDES IN RAT CHOLANGIOMA AND MOUSE HEPATOMA

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In previous communications [1, 2] we have presented results of studies on transamination of dicarboxylic amino acids and their amides in tissue slices from a number of α tumors and from malignant stomach tumor. Data from these experiments indicating a high activity of glutamic-oxalacetic transaminase are in accord with the high protein-synthesizing capacity of malignant tumor cells and with assumptions as to the leading role of transamination in amino acid conversions in the organism [3]. Added glutamine and asparagine are transaminated 2-16 times less rapidly than the corresponding dicarboxylic amino acids.

These results are confirmed in a study of Barban and Schulze [4] in which it was shown that glutamic acid is transaminated 9-16 times more rapidly than glutamine in cell-free extracts of Hela cells and of fibroblasts grown in tissue culture. The highly active glutamic-oxalacetic and glutamic-pyruvic transaminases have also been found in lymphosarcoma [5].

However, neither in our experiments nor in those of Barban and Schulze has it been determined whether there remain in tumor tissue specific enzyme systems for transamination of amides (glutamine and asparagine transaminases). Therefore, comparative studies of transamination of dicarboxylic amino acids and of their amides were made using rat cholangioma (strain PC-1) and a pure strain of mouse hepatoma (strain C_3HA). Livers of tumorous and of healthy animals served as controls.

METHODS

Fifty mg of tissue slices of 15 mg of lyophilized hepatoma preparations made according to Meister [6] were incubated under aerobic conditions in 0.2M tris buffer, pH 7.0 (in a number of experiments, in 1/15M phosphate buffer, pH 7.0) with 10 μmoles amino acid and 20 μmoles keto acid for 2 hours at 37 deg. Control samples contained only keto acid or only amino acid (sample volume, 1 ml). After incubation, protein was precipitated by addition of 0.2 ml 3M acetate buffer, pH 4.8, and boiling 4 minutes, after which it was removed by centrifugation. Amino acids in the supernatant liquid were determined by a modified method of Paskhina [7].

Thirty microliters of protein-free filtrate and neutralized standard solutions of glutamic and aspartic acid were place on Whatman No. 1 chromatographic paper. The solvent system employed was an n-butanol–formic acid–water (75:15:10) mixture. After five passages of the developing solution, the chromatogram was dried at room temperature and sprayed with 0.5% ninhydrin. A day later the amino acid spots were cut out and eluted for 1 hour with 5 ml of a saturated solution of $CuSO_4$ in 75% ethanol. Samples were periodically shaken and measured against a blank sample (eluate of paper through which the solvent had passed) on an FEK-M colorimeter or an SF-4 spectrophotometer at 510 mu.

Results were expressed in mg amino acid formed per g original tissue weight, the value for the control sample being deducted. Substrates employed were the L-isomers of glutamine, asparagine, alanine, glutamic acid, aspartic acid, and α-keto acids (ketoglutaric, oxalacetic, and pyruvic acids).

TABLE 1

Formation of Glutamic Acid in Rat Cholangioma and Rat Liver by Transamination and Reductive Amination of α-Ketoglutaric Acid. Glutamic acid in mg/g tissue

α-Ketoglutaric acid + asparagine			α-Ketoglutaric acid + aspartic acid			α-Ketoglutaric acid + NH_4Cl		
1*	2*	3*	1	2	3	1	2	3
Traces	0,97	2,03	2,63	3,12	2,67	0,44	0,76	0,84
0	1,30	2,70	1,30	4,31	3,76	0,27	0,57	0,73
0	3,05	—	1,10	2,19	—	0,35	0,63	—
Traces	1,20	—	1,41	3,52	—	0,40	0,60	—
0	1,70	—	1,07	3,76	—	0,24	0,49	—

*Here and in Table 2: 1—rat cholangioma; 2—liver of tumorous rats; 3—liver of healthy rats.

TABLE 2

Formation of Aspartic Acid in Cholangioma and Liver of Rat by Transamination and Reductive Amination of Oxalacetic Acid. Aspartic acid in mg/g tissue

Oxalacetic acid + glutamine			Oxalacetic acid + glutamic acid			Oxalacetic acid + NH_4Cl		
1	2	3	1	2	3	1	2	3
0,08	0,40	0,50	2,05	2,30	2,70	Traces	0,14	0,16
0,06	0,35	0,37	1,23	4,11	3,50	0	0,07	0,10
0,13	0,57	—	1,07	2,45	—	Traces	0,12	—
0,11	0,44	—	1,52	2,37	—	0	0,09	—
0,10	0,38	—	1,60	1,90	—	0	0,15	—

RESULTS AND DISCUSSION

In the study of asparagine-, glutamine-, and glutamic-oxalacetic transaminases, the samples to which asparagine and glutamine would be added were first heated 10 minutes at 50 deg and then rapidly cooled to 0 deg in order to inactivate the deamidases asparaginase 1 and glutaminase 1 [6]. Data obtained in these experiments are presented in Table 1.

As seen in Table 1, there is no formation of glutamic acid from asparagine in rat cholangioma, while in the livers of both tumorous and healthy animals, 1.64 and 2.36 mg/g tissue, respectively, is formed. On the other hand, aspartic acid increases glutamic acid formation in all tissues, although in cholangioma it is less than half that occurring in liver (1.5 mg compared to 3.38 and 3.21 mg in liver). Ammonium salt also activates glutamic acid synthesis in all tissues, but this is almost five times lower than with aspartic acid added. These data indicate that a specific asparagine transaminase is completely absent in tumor tissue, while a fairly active glutamic-oxalacetic transaminase is present.

Experiments on transamination of glutamine and glutamic acid (Table 2) revealed that glutamine does not enhance formation of aspartic acid from oxalacetic acid; this yield was approximately equal to that from α-ketoglutaric acid and NH_4Cl (0.09 and 0.11 mg). These data lead to the conclusion that cholangioma is lacking in a specific glutamine transaminase. The small increase in aspartic acid (0.09 mg) is probably due to a residual glutaminase 1 activity. In all three tissues, glutamic acid is vigorously transaminated with oxalacetic acid, the control rat liver exhibiting the greatest activity.

Since no substantial difference in transaminating activity of liver from healthy rats and liver from tumorous rats was observed, in the following series of experiments only mouse hepatoma (strain C_3HA) and liver of healthy mice was used. In contrast to the preceding experiments, these were performed with heated samples (50 deg for 10 minutes) as well as with the original tissues. Heating was carried out in order to inactivate glutaminase 1 and asparaginase 1. Results are presented in Table 3.

TABLE 3

Formation of Glutamic and Aspartic Acids in Mouse Heptaoma, Mouse Liver, and Lyophilized Extracts of Rat Cholangioma and Rat Liver by Transamination and Reductive Amination of α--Ketoglutaric and Oxalacetic Acids (Average Values)

Exptl. cond.	Inc. in glutamic acid, mg/g tissue						Inc. in aspartic acid, mg /g tissue					
	α-ketoglutaric acid + asparagine		α-ketoglutaric acid + aspartic acid		α-ketoglutaric acid + NH_4Cl		oxalacetic acid + glutamine		oxalacetic acid + glutamic acid		oxalacetic acid + NH_4Cl	
	1	2	1	2	1	2	1	2	1	2	1	2
Mouse hepatoma (1) and liver of healthy mice (2)												
Before eating	2.20	5.10	3.50	7.40	1.54	2.75	2.13	3.75	2.79	4.04	0.09	0.60
After eating	0.13	2.73	3.40	6.82	0.77	1.14	0.07	2.45	2.26	3.47	0.12	0.55
Rat cholangioma (1) and liver of healthy rats (2)												
Before eating	0	0.63	0.77	0.94	0	0.03	0	0.37	0.41	0.52	0	0.01
After eating	0	0.59	0.80	1.12	0	0.04	0	0.35	0.43	0.50	0	0.01

As previously reported [8], there are an active glutaminase 1 and asparaginase 1 in mouse hepatoma (samples before heating). After heating, neither asparagine nor glutamine enhances formation of dicarboxylic amino acids in this tissue. This indicates the absence of glutamine and asparagine transaminases. In the liver of healthy animals, heating does not substantially reduce formation of glutamic and aspartic acids from the corresponding amides by transamination; this confirms the finding of Meister [9] that there are specific glutamine and asparagine transaminases in liver. On the other hand, dicarboxylic amino acids are 2-3 times more active as amino group donors in transaminations with the corresponding α-keto acids in mouse liver tissue. Similar results were obtained by Barban and Schulze [4] with glutamine and glutamic acid in cell-free extracts of Hela cells and of mouse fibroblasts. In these experiments, however, possible participation of glutaminase 1 was not excluded; they ascribe the relatively weak participation of glutamine in transaminations with various α-keto acids to a slow deamidase action, and at the same time do not rule out the presence of a specific glutamine transaminase.

In tumor tissue, α-ketoglutaric acid undergoes reductive amination in the presence of NH_4Cl, while oxalacetic acid is almost untouched (Table 3). In normal liver tissue, the formation of aspartic acid from oxalacetic acid and NH_4Cl is 5-6 times as high as in hepatoma tissue. Thus, in tumor tissue reductive amination processes are either completely lacking or very insignificant, while transamination of dicarboxylic amino acids (but not their amides) is extremely rapid.

In the study of transaminase activity of lyophilized preparations of rat cholangioma and rat liver, enzyme preparations were heated 10 minutes at 50 deg to inactivate deamidases (Table 3). In contrast to the tissue slices, lyophilized preparations failed to exhibit reductive amination of added α-keto acids. With or without deamidase inactivation, asparagine and glutamine did not enhance amino acid formation in cholangioma, but did do so in liver. These results support the previous conclusion that in cholangioma, in distinction to liver, specific transaminases of glutamine and asparagine are absent. In tumor tissue, dicarboxylic amino acids are transaminated at almost the same rate as in liver (Table 3). Heating has no effect on transamination of dicarboxylic amino acids in either tissue, or on transamination of glutamine and asparagine in liver tissue.

Our data thus attest to the existence of an extremely active glutamic-oxalacetic transaminase in rat cholangioma and mouse hepatoma, and also to the absence of specific glutamine and asparagine transaminases. These results are in accord with those of Kit and Awapara [5] and Barban and Schulze [4]. The data indicating a higher glutamic-oxalacetic transaminase activity for liver than for tumor tissue are not in contradiction to previous findings of an active transaminase of dicarboxylic amino acids in malignant tumor tissues [1, 2]; calculation of amino acid formed per gram dry weight shows that the values are extremely close or even a little higher for tumor tissue than for liver.

An active glutamic-pyruvic transaminase has been found in regenerating rat liver by Pollak [10]. Studying this enzyme in embryonic tissues, Drel' [11] showed that during embryo growth enzyme activity rises. This con-

clusion is at variance with the results of Cohen et al. [12] who found that as a rule glutamic-pyruvic transaminase is absent in rapidly growing tissues, including tumor tissues.

These findings indicate that the problem of the specific properties of amino acid metabolism in tumor tissue cannot be considered solved.

CONCLUSIONS

In rat cholangioma and mouse hepatoma there is a highly active glutamic-oxalacetic transaminase. In mouse hepatome, asparagine and glutamine enter into transamination reactions with α-keto dicarboxylic acids only after hydrolysis to aspartic and glutamic acids by specific deamidases.

It has been established that specific glutamine and asparagine transaminases are absent in the tumor tissues studied. Reductive amination of oxalacetic acid does not occur, while that of α-ketoglutaric acid does, the process being several times less rapid in tumor tissue than in liver.

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BORON DISTRIBUTION IN VARIOUS FRACTIONS OF BRAIN AND LIVER TISSUE

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Boron was first found in animal tissues by Bertrand and Agulhon [1]. It had previously been found only in urine [2] and milk [3]. It was thought that the boron which animals obtained in plant foodstuffs was wholly excreted, not being incorporated by the organism.

Having demonstrated and quantitatively determined boron in various mammalian tissues, and also in tissues of many other organisms [1], Bertrand and Agulhon concluded that its occurrence in animals is as characteristic of them as of plants. The only difference is that in animal tissues it occurs in very insignificant amounts, these being in the order of $n \cdot 10^{-5} - n \cdot 10^{-6}$% of fresh tissue weight. Marine animals are capable of accumulating considerably more boron—up to $n \cdot 10^{-4} - n \cdot 10^{-3}$%. The data of Bertrand and Agulhon were confirmed by the investigations of other authors [4-9].

To a considerable extent, the amount of boron accumulated is determined by the amount entering the organism. With an increase of boron content of foodstuffs ingested by humans and other animals, there is a marked enrichment of their tissues [10-12]. Evidently this element is a necessary component of metabolic systems in animals. This has not been as clearly demonstrated as in plants, where exclusion of boron from the nutrient supply leads to sickness and death. It has proven practically impossible to rigidly exclude boron from animal diets since even carefully purified synthetic nutrients contained slight amounts of boron (~155 μg/kg) sufficient to satisfy the organism's requirements [13, 14]. Boron deficiency symptoms have been described by Skinner and Mc Hargue [11] for rats maintained on rations poor in boron and potassium. These symptoms are a shortening of the life span and a disappearance of body fat reserves and liver glycogen. To obtain these symptoms, it is important that boron and potassium be supplied in a definite ratio.

The physiological role of boron in animals is obscure. Only a few scattered data are available, and these are not sufficient to indicate the precise metabolic patterns involved. Considerably more is known for plants, the effect of boron on carbohydrate metabolism and to a lesser extent on protein metabolism, the interaction of boron with other mineral elements, and other problems having been well studied [15].

Another aspect which has been almost completely neglected is that pertaining to forms in which boron occurs in the organism. It is assumed that in the plant cell part is bound to the structural elements of the cell wall, the pectinaceous materials and carbohydrates [16, 17], while part is concentrated in the cell sap in soluble form [18]. Attempts to isolate organic boron complexes from plants have been unsuccessful [19]. In studies with animal tissues, Pfeiffer demonstrated the presence of boron in the acetone fraction of brain from dogs poisoned with boric acid [20]. He assumes that the organic compound in question is boroglycerate.

At present there is little information on the nature of the organic boron compounds in living tissues; such information is important in that it has a bearing on the physiological role of boron. In view of this, it seemed of interest to determine boron levels in various functions of animal tissues—protein, fat, and carbohydrate fractions. A study was made of sheep from northwestern Kazakhstan, an area rich in boron, where the excessive amounts in-

TABLE 1

Boron Distribution in Various Fractions of Brain and Liver Tissue. Boron content in mg%

Carbohydrate fraction		Fat fractions				Whole tissue	
		liver		brain			
liver	brain	acetone	ether	acetone	ether	liver	brain
1,77	0,41	0,0051	Traces	0,018	0,01	1,80	0,42
0,48	0,46	0,0074	"	0,0069	0,0022	0,52	0,49
0,60	0,30	0,0037	"	0,023	Traces	0,61	0,28
0,11	0,38	0,0039	0	0,0053	0,0084	0,10	0,37
0,28	0,17	0,009	0,0027	0,012	0,017	0,29	0,23
0,18	0,45	0,007	Traces	0,016	0,021	0,21	0,45
0,036	0,061	Traces	0	0,01	Traces	0,042	0,086
0,25	0,34	0,003	Traces	0,019	0,014	0,27	0,34
0,12	0,028	0,0042	0,0035	0,0053	0,006	0,12	0,054
Average 0,425	0,288	0,0048	Traces	0,0128	0,0087	0,440	0,302

gested induce specific illnesses in these animals [12, 21]. The boron enrichment of tissues under these conditions facilitated our study.

METHODS

Brain and liver tissues were fractionated. Proteins and carbohydrates were isolated prior to shipment, while tissue for fat extraction was fixed in 3% formalin and shipped in this form.

Preparation of material for protein and carbohydrate extraction. Tissues samples weighing 30-40 g were removed quickly after sacrifice of the animals. They were then minced and ground in a mortar with quartz and sand, and the slurries were mixed with the appropriate solvents.

Protein extraction. Tissue was extracted with three volumes (w/v) of physiological solution for one hour with constant shaking in flasks fitted with ground glass stoppers. As has been established, the major portion of protein in these tissues is extracted by this procedure. After standing, the extract was filtered through paper pulp on a Buchner funnel. The clear protein-containing filtrate was then transferred to another flask. Quantitative recovery was impeded by poor filtration of the proteins.

Carbohydrate extraction. During grinding of the tissue in a mortar, one and one-half volumes (w/v) 4% TCA was added. The slurry was filtered through an ordinary filter on a Buchner funnel. The residue was removed from the filter and reground, and the resulting slurry was filtered. This procedure was repeated until there was no reaction of the filtrate for carbohydrate (with α-naphthol).

Fat extraction. Acetone and ether fractions, which contain almost all the fats and fat-like substances present in the tissues investigated, were obtained in a Soxhlet apparatus. Extractions were carried out using tissue samples weighing ~30-40 g. Tissues were first dried by grinding with anhydrous Na_2SO_4. Individual fractions were washed and assayed for B. Unfractionated tissues were also analyzed. Boron was determined by the carmine method, being distilled as boromethyl ether [22].

RESULTS AND DISCUSSION

Analyses showed that there is no boron in the protein fractions of brain and liver. It is, however, present in the carbohydrate and fat fractions of both tissues. As the table shows, a large part is concentrated in the carbohydrate fractions. On the average, ~97% of the total amount in liver is bound to carbohydrates, while 95% of that in brain occurs in this form. The remainder us distributed in the acetone and ether fractions.

It has been reported that boron is able to form complexes with various polyhydroxy compounds [23]. Evidently its presence in the carbohydrate fractions can be explained by an ability to form this type of complex with carbohydrates and their derivatives. It is thought in some quarters that formation of such complexes in plants is a prerequisite to sugar transport, and also to synthesis and conversion of sugars [24-29]. It has not been conclusively demonstrated that boron exerts a direct action on these processes by, for example, formation of complexes with carbohydrates, but the disruption of such processes attendant on a boron deficiency or absence is clear evi-

dence that the element does affect these processes.

If it is assumed that the exsitence of boron in a given tissue fraction indicates a function in the organism, then, by analogy with plants, it may be concluded that boron participates in the carbohydrate metabolism of animals.

The insignificant amounts found in the fat fractions may be a consequence of the close relation of fat to carbohydrate metabolism, with various 3-C carbohydrates serving as precursors in fat synthesis.

SUMMARY

Boron is a necessary component of animal tissues. Although its physiological role is obscure, there is reason to believe that it is not unimportant in metabolism.

Almost all the boron of sheep brain and liver is associated with carbohydrates. About 95% of the total amount in brain is concentrated in this fraction, and about 97% of that in liver. The remainder is associated with the fat fraction. Protein fractions of these tissues contain no boron.

The presence of boron in carbohydrate fractions may be considered one of the proofs of its participation in carbohydrate metabolism in animals.

In conclusion, I wish to extent my sincere appreciation to Professor V. V. Koval'skii, who directed this study.

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THE EFFECT OF IRRADIATION ON MAGNESIUM CONTENT OF RAT LIVER

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In an earlier study [1] it was shown that whole-body irradiation of rats leads to a marked retardation of sedoheptulose-7-phosphate (S-7-P) utilization in rat liver extracts by stimulating fructose-1,6-diphosphatase activity. The well known fact that magnesium ions strongly activate fructose diphophatase suggested that the change in activity of this enzyme with irradiation may possibly occur under the influence of magnesium. The effect of irradiation on oxidative conversion of carbohydrates described in the previous study [1] would then be attributable to a change in magnesium concentrations in the organism, specifically, to an increase in the liver. To test this hypothesis it was necessary to see, first, whether addition of magnesium to control samples would induce the same disturbances in carbohydrate metabolism as were found in liver extracts of irradiated animals, and second, whether irradiation leads to an increased magnesium concentration in the liver. The results of investigations on these points are presented below.

METHODS

Experiments to determine the effect of Mg on the oxidative metabolism of carbohydrates were set up under the same conditions and with the same reaction components as previous experiments on the effect of irradiation on ribose-5-phosphate (R-5-P) cleavage [1]. Enzyme extracts and the necessary preparations were obtained as previously [1]. Determination of compounds under study was carried out by the methods described in [1].

Determinations were made using rat liver homogenates. Animals were sacrificed one day after whole-body irradiation with Co^{60} α-rays (distance from radiation source to center of animal body, 35 cm; dose rate, 17 r/min). For one day prior to the experiment, the animals were not fed. The liver was ground in a glass homogenizer in eight volumes of 0.15M KCl. The homogenate was assayed for Mg by the method with titanium yellow [2].

RESULTS

Data on the formation and decomposition of S-7-P are presented in Fig. 1. The difference between experimental and control samples is strikingly evident. In control samples, a rapid disappearance of previously formed S-7-P beginning at 60 minutes was noted, while in samples supplied with Mg no such disappearance took place. Thus, the effect of Mg on S-7-P utilization is analogous to that of irradiation [1]. Moreover, the effect of Mg is manifested consistently, and in a number of cases is more pronounced, than is that of irradiation. (Irradiation did not always completely inhibit S-7-P disappearance; in some experiments loss occurred, though less rapidly than in the control).

The effect of Mg on fructose diphosphatase was also analogous to the effect of irradiation: Addition of Mg caused a marked activation of the enzyme (Fig. 2) Patterns of R-5-P disappearance and hexosephosphate formation in the presence of Mg were similar in character to those observed in irradiated animals.

Thus, it seemed very likely that by such seemingly different treatments as irradiation and alteration of Mg levels it was possible to disrupt oxidative utilization of hexose in a specific way, i.e., by interference with the

Effect of Whole Body Irradiation on Magnesium Content of Rat Liver 24 Hours After Treatment

Irradiation dose r	Mg content, mg			Increase in Mg. conc. during irradiation, % of control*
	per g wet wt.	per g dry wt.	of whole organ	
No irradiation	0.54	1.85	3.67	
600	0.58	2.15	4.45	7.4
No irradiation	0.40	1.33	2.07	
600	0.45	1.44	2.96	12.5
No irradiation	0.64	2.06	2.53	
900	0.69	2.32	3.38	7.8
No irradiation	0.53	1.78	3.11	
900	0.57	1.99	3.94	7.5

*Control value, amt. of Mg in 1 g wet tissue from control animals.

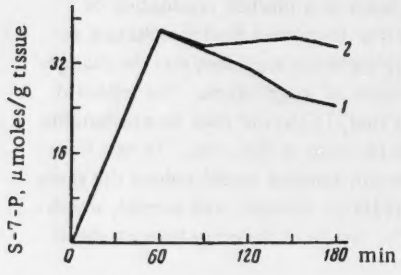

Fig. 1. Effect of magnesium on formation and loss of sedoheptuloso-7-phosphate. Composition of samples: 0.75 ml 0.25M glycyl-glycine buffer, pH 7.6; 0.3 ml liver extract; 6.6 μmoles R-5-P. Final vol. –3.75 ml. Incubation at 30 deg. 1 – –no Mg added; 2 –Mg added during incubation, 0.48 mg per ml incubation mixture.

action of fructose diphosphatase. This means that if it could be shown that irradiation leads to an increase in liver Mg, then it might be affirmed with some degree of certainty that this is one of the ways in which irradiation affects carbohydrate metabolism.

The table shows that whole-body irradiation in doses of 600 and 900r causes a significant rise in total liver Mg. This increase is also striking when calculated on the basis of tissue dry weight. Evidently, however, it is not total Mg content of the organ which is critical for enzyme activity, but the concentration in the system in which the reaction proceeds, i.e., amount per g wet tissue. As a rule, this was also higher in irradiated animals than in controls. It is striking that differences in liver Mg levels between irradiated and control animals are much less than those among individual control animals, used in separate experiments. The underlying causes of differences between control and irradiated animals in a single experiment, and between control animals of different experiments, are, however, far from identical. Check experiments carried out with several animals sacrificed at the same time and with a large number of parallel samples showed that within the limits of a single experiment variation in Mg content of the liver was slight, and that the method of determining Mg yielded values which agreed well from sample to sample. It is therefore clear that differences between control and experimental animals with respect to Mg content of liver are due to the effect of irradiation, and not to procedural errors. As far as the considerable divergence in values obtained in different experiments is concerned, they are evidently due to individual and seasonal variations.

At first glance, increase in Mg concentration of experimental samples seems slight; 7.4-12.5% of control (table). It should be taken into account, however, that in liver cells Mg occurs mainly in bound form, being a component of stable complexes with proteins of the nuclear, mitochondrial, and microsomal fractions [3]. Only about 13% of the total amount in the cell occurs in free form, or, more precisely, in the soluble fraction. An increase in Mg concentration even of the small absolute amount observed is therefore considerable in terms of its concentration in the soluble fraction, being as high as 100% (based on the figure 13% for free Mg). Since fructose diphosphatase is in the soluble fraction, it is natural that an alteration of Mg concentration in this fraction would affect the rate of fructose diphosphate cleavage. Thus, the at first glance insignificant increase in Mg concentration induced by irradiation proves on closer examination to be highly significant.

The method of Mg determination employed yielded information only as to sum total of free and bound Mg.

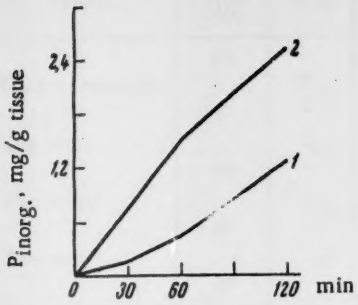

Fig. 2. Effect of magnesium on fructosediphosphatase activity in liver. Composition of samples 0.5 ml 0.25 M glycylglycine buffer, pH 7.6; 10 μmoles FDP; 0.2 ml enzyme preparation. Final volume, 2.5 ml. Incubation at 30°. 1) No Mg added; 2) Mg added during incubation, 0.48 mg per ml incubation mixture. a) $P_{inorg.}$, mg/g tissue.

Effect of Whole-Body Irradiation on Magnesium Content of Rat Liver 24 Hours After Treatment

Irradiation dose r	Mg content, mg			Increase in Mg conc. during irradiation, % of control*
	per g wet wt.	per g dry wt.	of whole organ	
No irradiation	0,54	1,85	3,67	7,4
600	0,58	2,15	4,45	
No irradiation	0,40	1,33	2,07	12,5
600	0,45	1,44	2,96	
No irradiation	0,64	2,06	2,53	7,8
900	0,69	2,32	3,38	
No irradiation	0,53	1,78	3,11	7,5
900	0,57	1,99	3,94	

*Control value, amt. of Mg in 1 g wet tissue from control animals.

However, it cannot be ignored that irradiation causes a pronounced rise in permeability of cellular and intracellular membranes as well as a disaggregation of complex structural entities. It is quite possible that this leads to a conversion of some portion of the eighty percent or more bound magnesium, possibly a very considerable portion, to free magnesium; thus, the actual increase in free magnesium of the soluble fraction would be considerably greater than that indicated by our calculations, which did not take this possibility into account.

DISCUSSION

A comparison of these data with previously reported data [1], has shown that the effect of irradiation on direct oxidative conversion of carbohydrates is similar to that of Mg, and in a whole series of cases is attributable to activation of fructose diphosphatase. In this connection the supposition arises that the effect of radiation in our experiments [1] is, primarily, to increase Mg concentration of the liver. Certain other changes occurring in the liver of irradiated animals can be explained in terms of such a phenomenon, for example, the increase in glycogen observed the first few days after treatment. As shown in experiments on white rats [4], intraperitoneal injection of Mg (as $MgSO_4$; 16 mg magnesium per 100 g body weight) leads to a pronounced rise in liver glycogen as early as one hour after injection. After 4 hours, the glycogen level has returned to almost the original value. Mg concentration of the plasma undergoes corresponding changes (marked rise in one hour, and return to normal in 4 hours). Thus, even with respect to synthesis and breakdown of liver glycogen, the effect of irradiation is probably mediated by alteration of liver Mg levels. The effect of Mg both on S-7-P utilization and hexose monophosphate formation might be due basically to a change in rate of phosphate cleavage of fructose diphosphate. An increase in Mg concentration during irradiation would therefore lead to rapid hydrolysis of this compound and to an associated increase in hexose monophosphate, which would in turn favor glycogen synthesis.

There are reports that an increase in fructose diphosphatase activity has accompanied the action of agents which stimulate glycogen synthesis [5].

SUMMARY

Whole-body irradiation of rats in doses of 600 and 900r brought about an increase in liver Mg.

Addition of Mg to extracts of liver from unirradiated animals results in the same interference with ribose-5-phosphate metabolism observed in extracts of liver from irradiated animals.

I wish to extend my sincere thanks to Professor S. E. Severin for directing this study.

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AN INHIBITOR OF THE SUCCINOXIDASE REACTION FROM Trypansoma cruzi

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In recent years there have been a number of reports of the isolation from various organisms of high-molecular-weight inhibitors of the succinoxidase reaction. Inhibitors of this type have been found in reticulocytes and mammalian tissues [1-3], in Tetrahymena piriformis [4], and in Hydra [5]. Protamines [6, 7] and carcinostatic antibiotics of a protein nature [8] are also able to inhibit tissue respiration. The existence of an inhibitor of the succinoxidase reaction in T. cruzi cells has been demonstrated by Trufanov and Palkina [9]. This study is devoted to the nature and mechanism of action of this inhibitor.

METHOD

A seven-day-old culture of T. cruzi grown on peptone-yeast extract medium [10] was centrifuged at 1800 g. The dense precipitate was washed out with a small amount of water and ground in a Waring blendor. The homogenate was then centrifuged at 1800 g, and the supernatant liquid (S) was used in experiments. The homogenate was prepared so as to yield S of such a volume that 1 ml corresponded to $2 \cdot 10^9 - 4 \cdot 10^9$ cells.

Crocker sarcoma tissue and mouse liver was ground in the cold in a Potter-Elvehjem homogenizer. The homogenate was then centrifuged at 800 g to yield a preparation free of cell debris and nuclei.

Determination of succinate-neotetrazolium reductase [11]. Composition of incubation mixture: 1 ml 0.1M phosphate buffer, pH 7.4; 0.5 ml 0.2M Na succinate; 0.2 ml 0.5% neotetrazolium chloride; 1 ml homogenate of sarcoma or normal liver tissue in 0.25M sucrose; 0.5 ml 0.9% NaCl. Experimental samples contained in place of the NaCl solution 0.5 ml S (1:10) or other test fraction. Samples were incubated at 37 deg for 20 or 40 minutes. The neobiformazan formed was extracted with 10 ml ethyl acetate and quantitatively determined at 530 mμ.

Determination of succinate-cytochrome c reductase [12]. The following reagents were place in a spectrophorometer cuvette: 1 ml 0.1M phosphate buffer, pH 7.4; 0.2 ml 0.3M Na succinate; 0.2 ml $2 \cdot 10^{-4}$M cytochrome c; 0.2 ml 0.02M KCN (neutralized with HCl); 1.2 ml H_2O. The reaction was begun by addition to the incubation mixture of 0.1 ml tissue homogenate prepared in 0.9% NaCl (1:10). The reaction was followed at 550 mμ, readings being taken every 30 seconds for 2 minutes.

Determination of succinic dehydrogenase [13]. Enzyme activity was determined by measurement of the rate of reduction of ferricyanide in the presence of succinate. Incubation mixture: 0.2 ml 0.1M phosphate buffer, pH 7.2; 0.2 ml 0.2M Na succinate; 0.3 ml 0.01M ferricyanide; 0.3 ml 0.1M KCN (neutralized with HCl). Upon addition of 0.1 ml tissue homogenate prepared in 0.9% NaCl (1:20), readings were taken on the spectophotometer at 400 mμ every 30 seconds for 2 minutes.

Determination of DPNH-cytochrome c reductase [14]. Rate of reduction of cytochrome c by DPNH was followed at 550 mμ. Incubation mixture: 0.2 ml 0.3M glycyl-glycine buffer, pH 8.5; 0.1 ml 1% cytochrome c; 0.1 ml 0.006M DPNH; 0.1 ml 0.1M KCN (neutralized with HCl); 2.5 ml H_2O. Tissue homogenate prepared in

Tissue	Incubation, min.	Additive	Neobiformazan formed μg
Crocker sarcoma	40	–	132
		S_{in}	60
The same	40	–	128
		S_{in}	84
The same	40	–	167
		S_{in}	105
The same	40	–	107
		S_{in}	48
The same	40	–	108
		S_{in}	42
Liver	20	–	195
The same		S_{in}	87
	20	–	180
The same		S_{in}	97
	20	–	196
The same		S_{in}	102

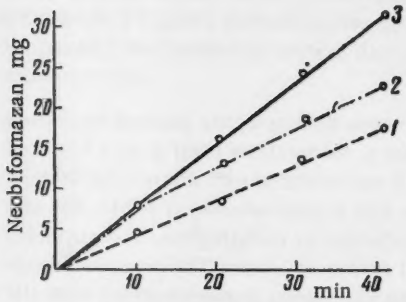

Fig. 1. Effect of preincubation of the homogenate with succinate on succinate-neotetrazolium reductase activity in the presence of an inhibitor from T. cruzi. Crocker sarcoma homogenates. 1 – succinate and S added together; 2 – homogenate preincubated 20 min. at 37 deg with succinate; 3 – S omitted.

0.02M $KHCO_3$ (1 : 10) was introduced into the cuvette in 0.05 ml aliquots, and the reaction was followed by readings every 15 seconds for 1 minute.

Determination of DPN-diaphorase [15]. Composition of incubation mixture: 0.3 ml 0.2M tris buffer, pH 7.5; 0.1 ml 0.006 DPNH; 0.1 ml 0.0012M 2,6-dichlorophenol indophenol; 2.5 ml H_2O. The reaction was begun by addition of 0.05 ml tissue homogenate in 0.2M tris buffer, pH 7.5 (1:40). The reaction was followed at 600 mμ with readings every 30 seconds for 3 minutes.

Determination of glucose-6-phosphate dehydrogenase [16]. Composition of incubation mixture: 0.2 ml 0.2M glucose-6-phosphate; 0.2 ml $1 \cdot 5 \cdot 10^{-3}$M TPN; 1.5 ml 0.04M glycyl-glycine buffer, pH 7.5; 0.2 ml 0.1M $MgCl_2$; 0.8 ml H_2O. Reaction was begun by addition of 0.1 ml tissue homogenate prepared in water (1 : 10). Readings were taken at 340 mμ every 15 seconds for one minute.

Determination of TPNH-cytochrome c reductase. To a system generating reduced TPN [16] were added 0.1 ml 1% cytochrome c and 0.1 ml 0.1M KCN (neutralized with HCl). Readings were taken at 550 mμ every 15 seconds for one minute.

Determination of glutamic dehydrogenase [17]. Enzyme activity was determined by measurement of rate of DPN reduction. Incubation mixture: 2.4 ml 0.005M phosphate buffer, pH 7.6; 0.1 ml 0.006M DPN; 0.1 ml homogenate prepared in water (1 : 10); 0.2 ml H_2O. Reaction was begun by addition of 0.2 ml 0.5M K glutamate. Readings were taken every 15 seconds for 1 minute at 340 mμ. In all spectrophotometric studies, the experimental samples contained in place of 0.1 ml water an equal amount of the fraction under investigation.

RESULTS

The effect of S on succinate-neotetrazolium reductase activity of Crocker sarcoma and normal liver homogenates was as follows: (See listing above)

These data indicate the presence of S of a substance which inhibits H^+ transfer from succinate to neotetrazolium. Subsequent investigation revealed that this substance is non-dialyzable and stable to heat. A 20-minute heating of S (pH 5.6-6.0) in a boiling water bath reduced inhibitor activity by only 10-20% (table). The inhibitor was stable over a wide pH range – from 3.0 to 8.0. At pH 2.0 and pH 9.0 partial inactivation occurred (table).

Preincubation of S with the homogenate did not enhance inhibitor activity. Preincubation of succinate with the homogenate reduced the inhibitory effect of S. Study of the dynamics of this process (Fig. 1) revealed that in the first 10 minutes of incubation reduction of neotetrazoiium was equally as rapid in samples with inhibitor and without it; subsequently, inhibition of the reductase is restored to the level characteristic of samples in which succinate and S were introduced simultaneously (parallel slopes of curves 1 and 2 in the 10-40 – minute time interval). Fig. 1 also shows that amount of neobiformazan formed in the presence of S is proportional to time of incubation in the 0-40 minute interval (curve 1).

The inhibitor could be quantitatively precipitated with 0.7 saturated $(NH_4)_2SO_4$ (pH 5.6-6.0). Curve 1 of figure 2 represents the activity of this precipitate.

With complete precipitation of inhibitor, the ratio of original (V_1) to final (V_2) volume should correspond to the ratio of inhibitor concentration in a sample containing undiluted inhibitor (S_1) to is concentration in a

The Effect of pH and of Heat on Activity of an Inhibitor of Succinate-Neotetrazolium Reductase. Crocker sarcoma homogenate. Incubation period – 20 min.

Additives	Neobiformazan formed, μg	Additives	Neobiformazan formed, μg
No additives	107	No additives	146
S	36	S	65
S, 60 min. at pH 2.0 (T 20 deg)	80	S, 60 min. at pH 2.0 (T 20 deg)	93
S, 60 min. at pH 9.0 (T 20 deg)	61	S, 60 min. at pH 9.0 (T 20 deg)	88
S, heated 20 min. at 100 deg.	44	S, heated 20 min. at 100 deg.	80

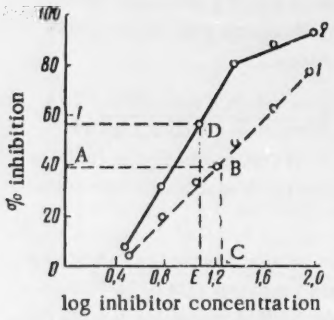

Fig. 2. Titration of inhibitor of succinate-neotetrazolium reductase from T. cruzi. Crocker sarcoma homogenate. 1 – inhibitor precipitated with 0.7 saturated $(NH_4)_2SO_4$, pH 5.6; original S volume (V_1) – 50 ml; volume after dialysis of redissolved precipitate (V_2) – 8.4 ml. 2 – inhibitor precipitated with 0.3-saturated $(NH_4)_2SO_4$, pH 4.6; original S volume (V_1') – 50 ml; volume after dialysis (V_2') – 7.8 ml. Final concentrations (S_1, S_2') of undiluted inhibitor from volumes V_2 and V_2' were taken as 100. Samples each contained 0.5 ml of inhibitor in a concentration series ranging from undiluted through 2-, 4-, 8-, 16-, and 32 fold dilution (corresponding to 2.0, 1.7, 1.4, 1.1, 0.8, and 0.5 on a log scale). Incubation period – 40 min. Remaining designations given in text.

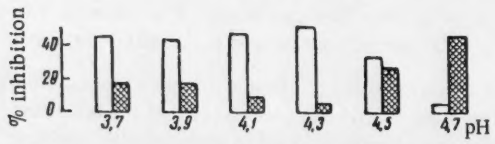

Fig. 3. Determination of isoelectric point of the succinate-neotetrazolium reductase inhibitor from T. cruzi. Crocker sarcoma homogenate. Five ml S were used for each determination. After adjustment of pH to the desired value, the precipitate was removed by centrifugation at 10,500 g and dissolved in the original volume of water. Dark bars – supernatant liquid; light bars – precipitate. Incubation time, 40 min.

sample of the original S (S_2). The original S inhibited reductase activity 38% (A on the ordinate axis). A line drawn horizontally from this point intersects the curve at point B. A perpendicular from this point to the abscissa (point C) intersects the axis at 1.24. The antilogarithm of 1.24 is 17.4 (S_2). $V_1/V_2 = 50:8.4 = 6.0$, and $S_1/S_2 = 100:17.4 = 5.8$.

These calculation indicte that the inhibitor is almost completely precipitated by $(NH_4)_2SO_4$ and is, therefore, either protein or some substance firmly bound to protein.

To determine the isoelectric point of the protein involved, either as the inhibitor itself or as a bearer of the inhibitor, S was acidified with dilute CH_3COOH or made alkaline with a weak solution of KOH. The precipitate was collected by centrifugation and suspended in the original volume of water. The resuspended precipitate and the supernatant liquid were then neutralized and tested for their inhibitory activity. The isoelectric point of the inhibitor was found to be pH 4.3 (Fig. 3). At this pH, the inhibition was almost completely precipitated. It should be pointed out that at pH's less than 4.3 (4.1-3.7), the inhibitor was not completely redissolved. Evidently this is related to poor solubility at ph < 4.3.

At a pH near 4.3, it is possible to completely precipitate inhibitor at low $(NH_4)_2SO_4$ concentrations. Curve 2 in Fig. 2 is a concentration-activity curve for a precipitate obtained by addition of 0.3-saturated $(NH_4)_2SO_4$ to S at pH 4.6 (fraction 0.3). Calculation similar to those performed for fraction 0.7 ($V_1'/V_2' = 50:7.8 = 6.41$; $S_1'/S_2' = 100:15.5 = 6.45$), show that in this case the inhibitor is quantitatively precipitated. All subsequent investigation were carried out with fraction 0.3, dialyzed to complete removal of $(NH_4)_2SO_4$ (sample with Ba^{++}). With this procedure, inhibitor concentration was consistently increased 5-6 – fold over that of S.

Kinetic analysis indicate that the inhibition is of the non-competitive type (Fig. 4). In Fig. 5, inhibition

of the reductase in Crocker sarcoma homogenate is compared with inhibition of that in normal liver. Evidently the difference between the tissues is due not to different inhibitor-enzyme relationships, but to differences in enzyme activity, this being lower in tumor tissue.

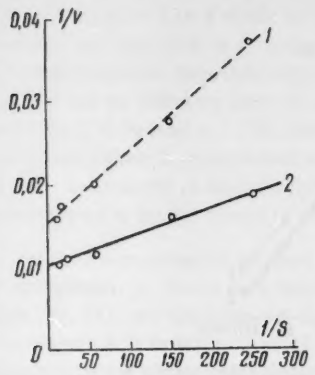

Fig. 4. Non-competitive inhibition of succinate-neotetrazoleum reductase by the inhibitor from T. cruzi. Crocker sarcoma homogenate: incubation time, 20 min. 1) In the presence of inhibitor; 2) without inhibitor. S—succinate conc. in mm; V—speed of the reaction in μg of neobiformazin.

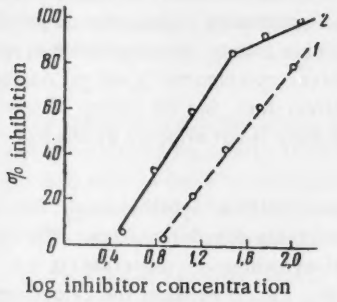

Fig. 5. Effect of various concentrations of inhibitor from T. cruzi on succinate-neotetrazolium reductase activity in homogenates of Crocker sarcoma and normal liver. Incubation time, 20 min. 1—liver; 2—Crocker sarcoma.

In order to pinpoint the side of inhibitor action in the biological oxidation system, we studied the effect of fraction 0.3 on various electron transport systems. These studies showed that the inhibitor completely suppresses succinate-cytochrome c reductase (Fig. 6A) and DPNH-cytochrome c reductase (Fig. 6B). It had little or no effect on rate of reduction of ferricyanide in the succinate $K_3Fe(CN)_6$ system (Fig. 6C). Glucose-6-phosphate dehydrogenase, glutamic dehydrogenase, TPNH-cytochrome c reductase, and DPN-diaphorase were unaffected. Vitamin K1, riboflavin-5-phosphate, EDTA, DL-α-tocopheryl phospate, adenylic acid, cysteine, and ascorbic acid all failed to relieve the inhibition of the succinate-neotetrazolium reductase.

Examination of the effect of α-tocopherol on the activity of succinate-neotetrazolium reductase inhibited by the unknown inhibitor revealed the following (Crocker sarcoma homogenate; incubation time 20 min.):

Tocopherol conc.	Additives	Neobiformazan formed, μg
	None	87
$\approx 5 \cdot 10^{-3}$ M	Fraction 0.3	30
	α-Tocopherol	85
	Fraction 0.3 + α-tocopherol	46
	None	47
	Fraction 0.3	20
$\approx 10^{-2}$ M	α-Tocopherol	49
	Fraction 0.3 + α-tocopherol	29

Thus, α-tocopherol relieved the inhibition caused by fraction 0.3 only partially. Since α-tocopherol was introduced into the incubation mixture in the form of an extremely unstable aqueous emulsion, determination of its final concentration was impeded, and this is therefore indicated only approximately.

DISCUSSION

The non-competitive inhibitor of succinate-neotetrazolium reductase from T. cruzi differs substantially from previously reported protein inhibitors of succinoxidase. The inhibitor from reticulocytes and mammalian tissues [1-3] reduces the activity not only of succinate-cytochrome c reductase and DPNH-cytochrome c reductase, but also that of succinic dehydrogenase succinate-phenazine metasulphate system) [18]. This inhibitor probably suppresses iron-containing flavins. This assumption is fully justified if it is taken into account that phenazine metasulphate evidently accepts H^+ directly from reduced succinic dehydrogenase [19] and that DPNH dehydrogenase and succinic dehydrogenase are iron flavoproteins [20, 21]. The succinoxidase inhibitor from T. piriformis [4] inhibits succinic dehydrogenase (succinate-phenazine metasulphate system) and DPNH-cytochrome c reductase. It also inhibits the dehydrogenation of glutamic and β-hydroxybutyric acids. Probably it is not narrowly selective in its action, therefore, but is effective in relation to a whole series of dehydrogenase systems. The T. cruzi inhibitor is similar in action to a protein from Hydra [5]. The latter has no effect on succinic dehydrogenase, but inhibits succinate-cytochrome c reductase. Unfortunately further comparison of the two inhibitors cannot be made, since no detailed description of the material from Hydra has yet been published. It should also be noted that the T. cruzi inhibitor is not a protamine. This is indicated by the following experimental data: The inhibitor is precipitated at acid pH's; it is activated at pH 2.0; it does not pass through a cellophane membrane.

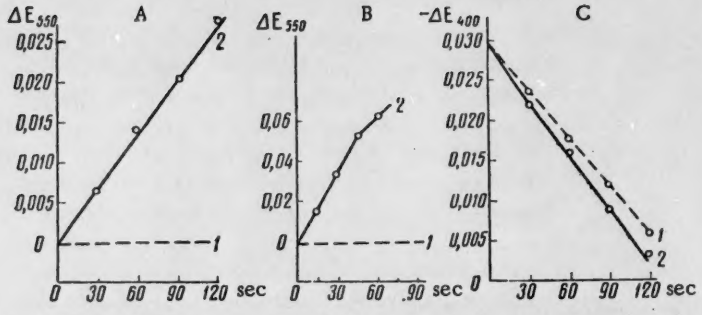

Fig. 6. Effect of the inhibitor from T. cruzi on activity of succinate-cytochrome c reductase, succinic dehydrogenase, and DPNH-cytochrome c reductase. A and C—Crocker sarcoma homogenate; B—liver homogenate. A and C—rate of reduction of cytochrome c by succinate; B—rate of reduction of cytochrome c by DPNH; C—rate of reduction by $K_3Fe(CN)_6$. 1—inhibitor present; 2—inhibitor absent.

The T. cruzi inhibitor suppresses cytochrome c reduction by succinate and by DPNH and neotetrazolium reduction by succinate. It has no effect on ferricyanide reduction by succinate. According to published information [13, 19], ferricyanide is reduced in the presence of succinate chiefly through the direct participation of succinate dehydrogenase. Therefore, in the presence of the T. cruzi inhibitor it is electron transfer from succinate to cytochrome c which is suppressed, and not the succinic dehydrogenase reaction. It should, however, be taken into account that according to Estrabrook [22], ferricyanide reduction by the enzymatic oxidation of succinate is partially inhibited by antimycin A. It might be assumed that ferricyanide plays a double role in the acceptance of H^+ during oxidation of succinate by cytochrome b and during eletron transfer between cytochromes b and c. At any rate, ferricyanide accepts H^+ at an earlier stage than cytochrome c and neotetrazolium, and the failure of fraction 0.3 to inhibit its reduction indicates that the rate of succinate dehydrogenation is not affected by this fraction.

Our data show that fraction 0.3 is without effect on electron transport from TPNH to cytochrome c. Evidently this process involves pathways other than those utilized in DPNH and succinate dehydrogenation. The following experimental data support this: TPNH oxidation mediated by TPNH-cytochrome c reductase (in distinction to DPNH-cytochrome c reductase) is insensitive to antimycin A [23, 24], i.e., it bypasses the cytochrome b - cytochrome c stage; when the transhydrogenase reaction is suppressed, oxidation of one molecule of TPNH is associated with formation of only one high-energy bond, in contrast to DPNH oxidation, where three bonds are formed [25, 26]. According to the scheme of Chance and Williams [27, 28], during DPNH oxidation phosphorylation occurs at the following stages—electron transfer from DPNH to flavoprotein, between cytochromes b and c, and between cytochromes c and a_3 (cytochrome oxidase). It may be supposed that electron transfer from TPNH as mediated by TPNH-cytochrome c reductase is a direct transfer to cytochrome c; under these conditions TPNH oxidation would be resistant to antimycin A action and would be associated with formation of only one high-energy bond.

Thus inhibition of succinate-cytochrome c reductase and DPNH-cytochromed c reductase by the T. cruzi inhibitor is accomplished at a point common to both systems, one which preceded cytochrome c. Since the inhibitor has no effect on the succinate dehydrogenase reaction, it may be supposed that its point of action is between cytochrome b and c, as for antimycin A [29]. This assumption finds support in the failure of the inhibitor to suppress the DPN-diaphorase activity; oxidation of DPNH in a system containing 2,6-dichlorophenol indophenol does not involve cytochromes b and c and is not suppressed by antimycin A [30]. The possibility that the inhibitor exerts an effect on cytochrome b is, however, not excluded, since recent data indicate that DPNH oxidation is associated with cytochrome b reduction [31, 32]. The recently isolated coenzyme Q, which functions between cytochromes b and c, participates in electron transfer from succinate and DPNH, and is sensitive to antimycin A [33], may be one of the sites of action of the T. cruzi inhibitor.

As yet there is nodecisive solution of the problem of the position of neotetrazaolium in the oxidation chain.

This problem is all the more pressing in that succinic dehydrogenase activity has been evaluated in terms of neotetrazolium reduction in a whole series of biochemical and particularly histochemical studies. It is known that neotetrazolium reduction in a succinate dehydrogenation system is sensitive to antimycin A [34, 35]. The T. cruzi inhibitor herein described suppresses neotetrazolium and cytochrome c reduction in a succinate oxidation system but has no effect on succinic dehydrogenase activity. Therefore, neotetrazolium functions at the level of cytochrome c or beyond it. This assumption is in accord with the views of other workers [34, 36], who believe that neotetrazolium is incorporated into the succinate oxidation system at the level of cytochrome oxidase. Evidently the mechanism of neotetrazolium reduction is different for succinate and DPNH dehydrogenation systems. This is attested to by the results of Slater [37, 38] and others [34,35,39].

In these experiements we have noted a partial relief of inhibition in the succinate-neotetrazolium system by α-tocopherol. It should be pointed out that it does not relieve the inhibitory action of antimycin A on succinoxidase [40, 41], and therefore the finding of Nason et al. [42] of a competitive interaction between α-tocopherol and antimycin A is in error. According to recent reports [43], α-tocopherol restores surface structure of individual components of the biological oxidation system which have been disrupted by the action of certain organic solvents We are disinclined to believe that itspecifically relieves succinoxidase inhibition caused by the T. cruzi inhibitor. Unfortunately, we do not yet have detailed information which would enable us to elucidate the mechanism of interaction between α-tocopherol and the T. cruzi inhibitor.

In summary, it is concluded that the inhibitor has a mechanism of action similar in many ways to that of antimycin A. In contrast to antimycin A, fraction 0.3 does not interfere with electron transfer from succinate to ferricyanide, and its inhibitory action is partially relieved by α-tocopherol.

SUMMARY

Homogenates of Trypanosoma cruzi cells contain a substance which inhibits the succinoxidase reaction in the Crocker sarcoma and in normal liver. The inhibitor reduces the activity of succinate-cytochrome c reductase, DPNH-cytochrome c reductase, and succinate-neotetrazolium reductase without affecting succinic-dehydrogenase, DPN-diaphorase, glutamic dehyrogenase, TPNH-cytochrome c reductase, and glucose-6-phosphate dehydrogenase.

The inhibitor is non-dialysable and is quantitatively precipitated by $(NH_4)_2SO_4$ at 0.3 saturation (pH 4.6). It is also precipitated by acidification of the homogenate to pH 4.3 A 20 min exposure of the homogenate to 100° (pH 5.6-6.0) reduces inhibitor activity by only 10-20%. At pH 2.0 and 9.0 partial inactivation of the inhibitor is noted. The inhibitor of succinate-neotetrazolium reductase and of electron transfer from succinate and DPNH to cytochrome c is either a protein or a compound firmly bound to proteins.

α-Tocopherol partially relieves inhibition of the succinoxidase. The succinate-neotetrazolium reductase inhibition is not competitive with regard to succinate. The most probable site of action of the inhibitor is either cytochrome b or some electron transfer agent intermediate between cytochromes b and c.

The mechanism of DPNH and TPNH oxidation, the site of activity of neotetrazolium in the oxidative chain, and the similarities and differences between the T. cruzi inhibitor and antimycin A are discussed.

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ENZYMATIC DECOMPOSITON OF UREA IN AN OPEN SYSTEM

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The study of enzymatic reactions in open systems is of interest for the theory of enzyme action in living cells, since all biological systems are open ones because of the continuous exchange of metabolites and energy with the surrounding environment. In a previous study [1], the steady state condition of an enzymatic reaction was investigated using as a model the oxidation of ascorbic acid by peroxidase, with kinetic and diffusion parameters being varied. It was shown that an enzymatic reaction in an open system has a number of characteristics which are absent in a closed system: a) In an open system, enzymes affect not only reaction rate, but also the apparent "equilibrium" position, i.e., steady state concentrations of the reactants; b) with alteration of enzyme concentration, rate of diffusion, or temperature, the system shifts from one steady state to another; c) within certain limits, systems have the capacity for dynamic stabilization of the steady state condition after passage through an intermediate maximum or minimum. In the study referred to above, quantitative limits of the system's dynamic stability were also determined.

This is a report on a study of urea decomposition mediated by urease in an open system. The products of this reaction are gases, $CO(NH_2)_2 \rightarrow CO_2 + 2NH_3$, so that the main component of the reaction solution is a single material—urea; during ascorbic acid oxidation, on the contrary, the solution contains two components—ascorbic and dehydroascorbic acids. This is the basis of certain specific properties of the reaction in question.

In general form, the condition of a two component solution in which a reaction is taking place is described by a pair of equations [2]:

$$a - \bar{a} = \mu_{1a} e^{-\lambda_1 t} + \mu_{2a} e^{-\lambda_2 t}$$
$$b - \bar{b} = \mu_{1b} e^{-\lambda_1 t} + \mu_{2b} e^{-\lambda_2 t}, \quad (1)$$

where $\bar{a}$ and $\bar{b}$ are the steady state concentrations of components A and B; a and b the concentrations of these components at a given moment of time; μ, constants; and λ_1, λ_2, roots of the equations. As time t increases, the right half of the equations tends to zero; this means that with time concentrations a, b reach the steady state values $\bar{a}$, $\bar{b}$. Depending on experimental conditions, this is described by three main types of curve (Fig. 1): change with passage through a minimum (1), simple transition to another steady state condition (2), and change with loss of the steady state condition (3). In two-component solutions or more complex ones, because of various combinations of constants μ and λ, all three types of change may occur, as shown experimentally [1]. For a one-component solution equations 1 reduce to the expression:

$$a - \bar{a} = \mu e^{-\lambda t}. \quad (2)$$

This system should be capable only of establishment of a steady state condition, but not of passage through a maximum or minimum, a point for experimental confirmation. In addition, we studied the effect of enzyme inactivation on the establishment of the steady state, since this was not investigated previously. This is a report of our findings on these two problems.

METHODS AND RESULTS

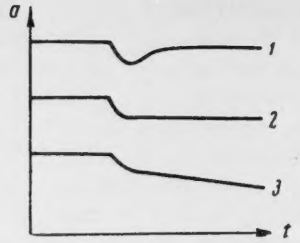

Fig. 1. Kinetics of change in reactant concentration at non-steady state conditions. Explanations in text.

Chemically pure urea was used; the urease preparation was obtained from soybean meal (activity indicated below). Urea concentration was determined according to Sanshe [3], but with $NaNO_2$ concentration altered to 0.002%, which made it possible to determine urea in 5-15μg amounts; a change from 5 to 15 μg urea corresponded to a change in extinction of the final yellow solution of from 0.420 to 0.240.

Measurements were made in a device described previously [1], into which flowed a 3% urea solution at the rate of 3.3 ml per hour. Volume of the reaction mixture was 100 ml. This was separated by a cellophane membrane from an external circulating buffer solution; membrane area was 100 cm^2. The external phosphate buffer bathing the membrane had a pH of 7.0, μ of 0.5M, and the internal solution in the reaction vessel contained urea (0.3%) in the same buffer. After establishment of the steady state, based on a dynamic balance between urea inflow and its diffusion out through the membrane, various amounts of enzyme were added to the internal solution, and the change with time of urea concentration was measured. In the course of the experiment, which lasted several hours, pH was held constant. All experiments were carried out at 30 deg. Measurement of diffusion of urea through the membrane (thickness 0.03 mm) showed that $D = 3.8 \cdot 10^{-7}$ cm^2/sec (average of five measurements). It was shown that decomposition of urea by urease is a zero order reaction (experiments performed for 0.3-, 0.7-, and 1.5% urea with 10, 25, and 50 mg enzyme preparation in 100 ml), as previously reported [4]. On the average, 1 mg enzyme preparation decomposed in 1 hour 0.64 ± 0.07 mg urea (average of 16 determinations).

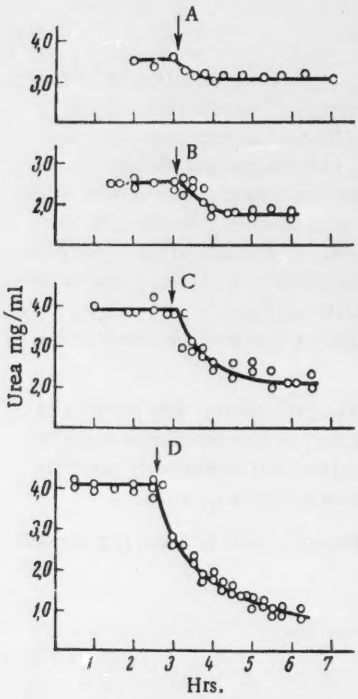

Fig. 2. Change in steady state concentration of urea after addition of various amounts of enzyme. Urease added: A – 25; B – 50; C – 100; D – 250 mg

Thus, the material balance of the system was as follows. Urea entered the system at the rate of 100 mg/hour; it diffused through the membrane at the rate of 60 mg/hour; 7.2 mg/hour was lost by removal of samples. Therefore, in the steady state condition, when the inflow of urea is exactly balanced by its total loss from the system, 32.8 mg/hour were utilized in the chemical reaction, i.e., the system worked with an efficiency coefficient of 32.8%.

In every experiment, the steady state concentration of urea was first established in the absence of enzyme (in 2-3 hours), and then the enzyme preparation was added and its effect on the establishment of a new steady state studied. Fig. 2 illustrates results of experiments in which 25, 50, 100, and 250 mg enzyme preparation were added at times indicated by arrows. With enzyme addition, the system passes to a new steady state without going through a maximum or minimum first. With addition of 25 mg enzyme, the system has arrived at a new steady state in an hour, with addition of 100 mg, ~3 hours are required, and with addition of 250 mg the system evidently fails to establish a new steady state. With two successive additions of 50-mg aliquots (Fig. 3), a repeated establishment of the steady state was observed, 2-3 hours being required each time and passage through a minimum being absent.

In later experiments, the effect of enzyme inactivation by a $AgNO_3$ solution on the change in steady state was studied. Degree of urease inactivation under static conditions at 30 deg by various concentrations of $AgNO_3$ is shown in Fig. 4. At $3 \cdot 10^{-6}$N $AgNO_3$, there is 50% inactivation, and at 10^{-5}N $AgNO_3$, inactivation is complete. Experiments were performed with these two concentrations under dynamic conditions; $AgNO_3$ concentration was kept constant by addition of an amount to the external buffer solution to give the same concentration as that of the reaction solution (in these experiments, citrate buffer, pH 5.75 used). Upon establishment of the initial steady state, 100 mg enzyme was added, and a second steady state was attained after 2.5 hours. The necessary amount of $AgNO_3$ was then added to the internal solution, and at the same time the external buffer solution was replaced. As Fig. 5 shows, addition of inhibitor leads to partial or complete restoration of the original steady state depending on the degree of enzyme inactivation.

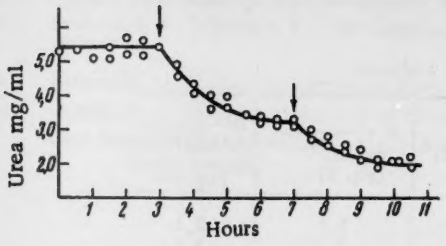

Fig. 3. Change in steady state concentration of urea after two successive additions of enzyme. First and second additions – 50 mg aliquots of urease.

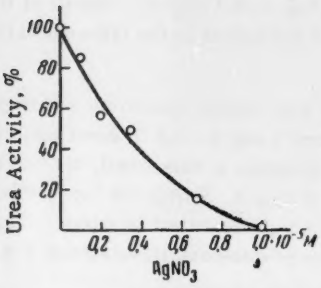

Fig. 4. Inactivation of urease by $AgNO_3$.

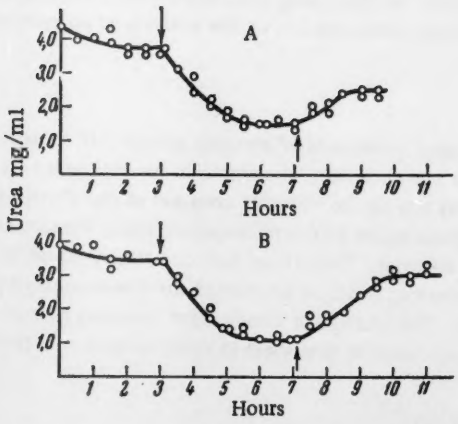

Fig. 5. Effect of urease inactivation by $AgNO_3$ on change in steady state. First additon, 100 mg urease (A and B). Second addition, $3 \cdot 10^{-6}$M $AgNO_3$ (A); 10^{-5}M $AgNO_3$ (B).

DISCUSSION

The study of enzymatic decompositon of urea in an open system reveals the emergence of a steady state condition based on continuous restoration of the system's composition and on the dynamic balance between inflow and outflow. In our experiments, urea was introduced into the system at a constant rate, and was lost from the system by diffusion through a membrane. Therefore, the change in urea concentration in the reaction vessel is described by the equation:

$$\frac{da}{dt} = K_0 - K_2 (a - a_z), \tag{3}$$

where K_2 is the constant for transfer or urea through the membrane; a, urea concentration in the vessel; a_Z, urea concentration in the external buffer. In our experiments $a_Z = 0$, and therefore

$$\frac{da}{dt} = K_0 - K_2 a, \tag{4}$$

Integrating equation 4, we obtain

$$a = \frac{K_0}{K_2} + \left(a_0 - \frac{K_0}{K_2}\right) e^{-K_2 t} \tag{5}$$

where a_0 is urea concentration at t = 0. Equation 5 describes the kinetics of the establishment of the steady state (first part of curves in Fig. 2, 3, and 5 before enzyme addition) and shows that after a sufficiently long time ($t = \infty$ in our experiments; after 2-3 hours) the second term on the right goes to zero. The steady state concentration $\bar{a}$ is thus described as:

$$\bar{a} = \frac{K_0}{K_2}. \tag{6}$$

Upon addition of enzyme, the decrease in urea in the internal solution rises to the value K_1, since the reaction in question is a zero order one. Change in concentration after addition of enzyme is therefore described as follows:

$$\frac{da}{dt} = K_0 - K_1 - K_2 a. \tag{7}$$

Integrating equation 7 and noting that at t=0, $a = \bar{a}$ (since addition of enzyme occurred after attainment of the initial steady state), we obtain

$$a = \frac{K_0 - K_1}{K_2} + \frac{K_1}{K_2} e^{-K_2 t}. \tag{8}$$

In this case the new steady state condition $\bar{a}'$ is defined by the equation

$$\bar{a}' = \frac{K_0 - K_1}{K_2}. \tag{9}$$

Using the values for K_0, K_1, and K_2 obtained experimentally, we calculated by equations 6 and 9 the steady state concentration of urea $\bar{a}'$ and compared it with experimentally obtained values of Figs. 2 and 3 (table).

TABLE

Calculation of Urea Steady Concentration after Enzyme Addition

Amt. of enzyme, mg	K_0, mg/hours	K_1, mg/hours	$\bar{\alpha}$ mg/ml	$K_2 = \frac{K_0}{\bar{a}}$, mg/hours (eq. 6)	$\bar{a}' = \frac{K_0 - K_1}{K_2}$, mg/ml (eq. 9)	$\bar{\alpha}'$ – experimentally obtained value, mg/ml
25 (Fig. 2)	100	16	3,5	29	2,9	3,1
50	100	32	3,2	31	2,2	2,4
50 (Fig. 2)	100	32	2,5	40	1,7	1,7
100 (Fig. 2)	112	64	3,9	28	1,6	2,1
50	100	32	5,4	18	3,8	3,3
+ 50 (Fig. 3)	100	64	—	18	2,0	2,1

Thus, for example, K_0 at an inflow rate of 3.3 ml 3% urea per hour is 100 mg/hour; K_1 for 25 mg enzyme (0.64 mg urea decomposed per mg enzyme per hour) is 16 mg/hour; $\bar{a}$ for the experiment with 25 mg enzyme is 3.5 mg/ml (Fig. 2); from equation 6, $K_2 = K_0/a = 29$ mg/hour. Therefore, from equation 9, $\bar{a}' = (K_0 - K_1)/K_2 = 2.9$ mg/m.; this compares with the experimentally obtained value for $\bar{a}'$ shown in Fig. 2, 3.1 mg/ml. Results of similar calculations are shown in the remaining lines of the table. Inspection of the figures in the table shows that experimental data agree quite well with calculated values.

In contrast to the ascorbic acid oxidation system studied earlier [1], the urea–urease system showed no passage through a maximum or minimum, which confirms deductions from equations 1 and 2. Fig. 2 shows that even at 25 mg enzyme, under conditions where the difference in steady state concentrations is very small, there is a simple shift from one steady state to another of the type described by curve 2 in Fig. 1. Within the limits of experimental error, in the one-component system under study no transitions of the type described by curve 1, Fig. 1, are observed. In two-component systems or more complex ones, all three types of transition illustrated in Fig. 1 are observed; they are also found in living organisms [5].

Our experiments have shown that enzyme inhibitors can affect the shift from one steady state condition to another. Inactivation of the enzyme by $AgNO_3$ caused the return of the system to the original steady state condition either partially or completely, depending on the extent of inactivation (Fig. 5); without doubt other modes of enzyme inactivation would have a similar effect. This effect of inhibitors is evidently peculiar to open systems; in closed systems, they are unable to bring about the original steady state condition. Superficially, the curves of Fig. 5 are similar to curve 1, Fig. 1, but in this case attainment of a steady state is due not to dynamic properties of the system itself, but to a new factor–enzyme inhibitor. In the living cell, inhibitors of various kinds (acting by chemical or absorptive means) may, by altering the actual concentration or the activity of an enzyme, serve to maintain steady state conditions.

SUMMARY

Urea decomposition was studied in the presence of urease under conditions of an open system (efficiency coefficient of the system 32.8%). Theoretically, steady state urea concentration was shown to be dependent on the transfer constants between the reaction medium and the system and on the velocity constant of the chemical reaction (equations 5 and 8). It was shown that the calculated values agree well with experimentally determined values for steady state urea concentrations at varying amounts of enzyme. Transitions between steady states in the system under study do not involve intermediate maxima or minima, which is accounted for theoretically by the one-component compostion of the system (equations 1 and 2). The change in steady state upon enzyme inactivation by $AgNO_3$ solutions was followed, and the specific significance of inhibitors in open systems as a factor in return of the system to the original steady state was indicated.

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All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of this issue.

ON THE INTERRELATIONSHIP BETWEEN NITROGEN FIXATION, RESPIRATION AND OXIDATIVE PHOSPHORYLATION IN *Azotobacter vinlandii*

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There are no definitely established opinions on the dependence of nitrogen fixation on the energy of respiration and oxidative phosphorylation. Some authors [1, 2] suggested that the reduction of inert molecular nitrogen by Azotobacter appears to require a supply of energy which is formed through the oxidation and phosphorylation of substrates. There are, however, also indications that nitrogen fixation can be accomplished without the expenditure of exogenous energy [3-5].

The reason for the discrepancy of those opinions lies in the attempts to investigate this most important phenomenon in cell-free systems, which are not suitable for the study of nitrogen fixation, since this process requires certain intact cellular structures for normal functioning [6-8]. The study of the dependence of nitrogen fixation on oxidative phosphorylation through the use of specific poisons on the intact bacterium was also rendered difficult due to the very low P/O ratios obtained under those conditions [9, 10].

The present communication deals with the development of an experimental procedure for the assay of the total phosphate esterified by intact Azotobacter cells as a result of oxidation of medium substrates. Having developed the method for assaying P/O ratios, we attempted to elucidate the possible dependence of nitrogen fixation on respiration and oxidative phosphorylation in intact cells of Az. vinelandii grown in a complete nutrient medium. The study of this problem in the present work employed a variety of inhibitors of respiration and of oxidative phosphorylation (DNP, NaN_3, KCNO), as well as ionizing radiation (X-rays), with the aim of producing a defined degree of uncoupling of the two processes and observing any possible changes in nitrogen fixation under the altered conditions. A comparative study of the three processes, in Azotobacter cultures grown on either molecular or ammonia nitrogen, may also be expected to yield some information on the nature of nitrogen fixation.

METHOD

The organism used was Az. vinelandii (from the Museum of the Dept. of Microbiology, Moscow State University); it was grown in a clear modified medium of Bourke [11], and harvested in the logarithmic phase (24 hours). Cells harvested for analysis were washed with fresh culture medium and re-suspended in fresh medium to give a suspension density of x 5-7 x 10^9 cells per ml.

Cell densities in normal cell suspensions were estimated nephelometrically; in suspension of cells treated with chloramphenicol or with ionizing radiation counting was carried out according to Vinorgadskii, since under those conditions cell division is affected, which results in increased cell size and leads to inaccuracies in the nephelometric assay.

The assay of respiration and oxidative phosphorylation in intact cells of Az. vinelandii. Cell respiration was measured manometrically as oxygen uptake in small (20 ml) Warburg vessels shaken with a frequency of 100 per minute at 30 ± 0.01°. Each vessel received two ml of the bacterial suspension equivalent to 10-15 x 10^9 cells.

TABLE 1

Factors Affecting Respiration and Oxidative Phosphorylation in Suspension of Azotobacter vinelandii

Expt. No.	Experimental conditions	Remarks	O_2 uptake per hr	P esterified per hr	P/O
			μg atoms/10^9 cells		
1	24-hour culture; Gas phase: air or oxygen. P uptake measured as orthophosphate disappearance from medium.	air	3,55	0,04	0,011
			3,55	0,04	0,011
		oxygen	11,50	0,10	0,009
			13,80	0,17	0,012
			11,60	0,17	0,014
2	30-hour culture. P-uptake as above	oxygen	6,20	0,05	0,008
			6,85	0,05	0,007
			5,95	0,05	0,008
	26-hour culture. Conditions as above	oxygen	11,40	0,05	0,004
			10,40	0,05	0,005
			10,10	0,05	0,005
3	24-hour culture, medium containing varying amounts of phosphate: 1000 mg P/liter (normal),	normal, 60 min	4,94	0,03	0,006
			5,40	0,03	0,006
			5,10	0,05	0,010
	104 mg/liter [17] and 325 mg/liter [10]. P uptake measured directly as increase in organic phosphorus of the cells. P^{32} was incorporated for 15, 30 and 60 min.	accord. to Ochoa* 15 min	2,4	0,15	0,063
			2,4	0,31	0,111
		30 min	2,4	0,10	0,042
			2,4	0,17	0,049
		60 min	2,4	0,02	0,009
		according to**			
		15 min	3,18	0,15	0,049
		30 min	3,18	0,08	0,025
		60 min	3,18	0,03	0,009
			3,18	0,02	0,006
4	24-hour culture. The cells were deprived of phosphorus (in a P-deficient medium) for 18 hr, followed by enrichment with P for one hour. P uptake estimated by measuring decrease in medium orthophosphate.	Normal	2,22	0,04	0,017
			2,50	0,04	0,016
		"Starved"	1,38	0,06	0,044
			1,48	0,06	0,047

*[17]. **Roberts [10]

Oxygen uptake readings were taken over a period of one hour, at 15-min intervals. Phosphorus was estimated by the method of Berenblum and Chain, as modified by Weil-Malherbe and Green [12].

In a number of experiments (Tab. 1) the phosphorus uptake was measured as the amount of inorganic phosphate disappearing from the medium. All results of oxidation and phosphorylation were expressed as μatoms of oxygen and phosphorus taken up per 10^9 Azotobacter cell per hour. The P/O ratios were calculated from those data.

Preliminary experiments showed that the rate of oxygen uptake depended on the age of the culture from which the cells were harvested, on the number of cells used, the composition of the gas phase (partial pressure of oxygen), the rate of shaking, the temperature of the bath etc. (Table 1); consequently, in all subsequent experiments standard conditions were strictly adhered to.

Measurements of respiration and of oxidative phosphorylation in Azotobacter over a period of one hour by the methods described above showed that the P/O ratio in this organism had very low values (Table 1). As had been established, Azotobacter has a powerful oxidative system [13-15], as well as an excellent capacity for oxidative phosphorylation [16-19]. The published values [2, 16, 18] for P/O ratios in Azotobacter are high, ranging from 0.5 to 1.0, but those were obtained only with subcellular fragments. It is also known that nitrogen fixation does not proceed well in cell-free preparation of Azotobacter [6-8]. An accurate quantitative estimate of the esterified phosphorus in whole cells is therefore essential for the study of the interrelationship between nitrogen fixation and oxidative phosphorylation. It should be mentioned that no reports dealing with the accurate determination of P/O ratios in intact Azotobacter cells have appeared in the literature.

TABLE 2

The Dependence of Observed Phosphorylating Intensity (P/O Ratio) on the Experimental Period of P^{32} Incorporation by Whole Cells of Az. vinelandii

Expt. No.	15 sec	1 min	15 min	30 min	60 min
1	1,140	0,320	0,063	0,042	0,009
2	1,510	0,109	0,049	0,025	0,005
3	1,135	0,400	0,051	0,041	0,006
4	1,810	0,400	0,050	0,035	0,009
5	1,040	0,109	0,063	0,038	0,007

Satisfactory estimates of P/O ratios with intact cells could not be obtained by measurement of the rate of inorganic P^{32} incorporation into the acid-soluble organic phosphorus [17], or into the entire organic phosphorus fraction of the cells, including the acid-insoluble portion (cf. Expts 1 and 2, Table 1). Variation of the total phosphorus content of the medium which was adjusted to contain, in different instances, 104 mg P per liter [17], 326 mg/liter [10] (cf. expts 3 and 4, Table 1) or the exposure of the cells to P^{32} for varying periods of time (15, 30 and 60 min – expt. 3, Table 1), also failed to alter the observed P/O ratios. Elevated values of P/O ratios – about twice those found in normal suspensions – were obtained with cells which were temporarily deprived of phosphorus by placing them in a phosphorus-deficient medium before the experiment, and by subsequently enriching the medium with phosphorus during the actual incubation [20] (cf. Expt. 5, Table 1). However, even this elevated value was still quite low (0.045) for an authentic value in intact cells. The reinforcement of the incubation media with catalytic amounts of ADP and with a trapping system (glucose and hexokinase [2]) was also of no avail, presumably because of the very poor permeability of the cell membrane to those compounds.

The low P/O ratios (0.050) found with the intact cells was, apparently, due to the high turnover rate of the high-energy phosphates and to a peculiar equilibrium between ADP esterification and the mineralization of the terminal P of ATP during the high rate of respiration. In the disrupted cell, where the overall cell harmony no longer holds, there is a certain disproportion between the formation of high-energy compounds and their expenditure for synthetic and other purposes, and this made possible the observation of higher experimental values for the P/O ratio (0.5-1.0) whenever oxidation and phosphorylation have not been seriously uncoupled in the given preparation.

On the basis of the above assumption we have checked the kinetics of radiophosphorus incorporation into the organic fraction after exposures to the isotope for 15 sec, 1, 15, 30 and 60 min. It was found that the experimentally observed and calculated value of P/O increased considerably with the decreasing experimental period of exposure to the isotope (Table 2).

A detailed analysis, using ion-exchange chromatography [21], of the various acid-soluble phosphorus compounds and of their radioactivity showed that, in the short-term experiments, a small amount of radioactivity was incorporated into ATP in the 15-sec experiment, and in both the ATP and ADP in the 25-sec experiment. In subsequent experiments P^{32} incorporation was measured exclusively over the initial 15-sec period, during which ATP only was labelled, and only terminally. The increased observed P/O ratios (1.0-1.5) in whole cells under such experimental conditions were simply due to the fact that the terminal phosphate acquired by the ATP in such a short time period could not be transferred to other acceptors to any appreciable extent, and thus the radioactivity acquired reflected the true phosphorus uptake during the interval. Indeed, the prominently ubiquitous active nucleoside diphosphokinases of Azotobacter [22, 23] would be quite capable of carrying the radioactive phosphorus to a range of further accptors in any more extended time [21].

In view of the above-said, an "instant" method was developed for the estimation of oxidative phosphorylation in whole Azotobacter cells. The method, described in detail below, has yielded reliable results.

After the completion of the suitable one-hour experiments in the Warburg vessels, designed for the assay of oxygen uptake and nitrogen fixation, under normal conditions and in the presence of various inhibitors, the bacterial suspensions were used for the assay of oxidative phosphorylation. For this purpose one ml of the suspension was added to one ml of the incubation medium, containing 70,000 cpm of P^{32} (as $K_3HP^{32}O_4$), in a centrifuge tube

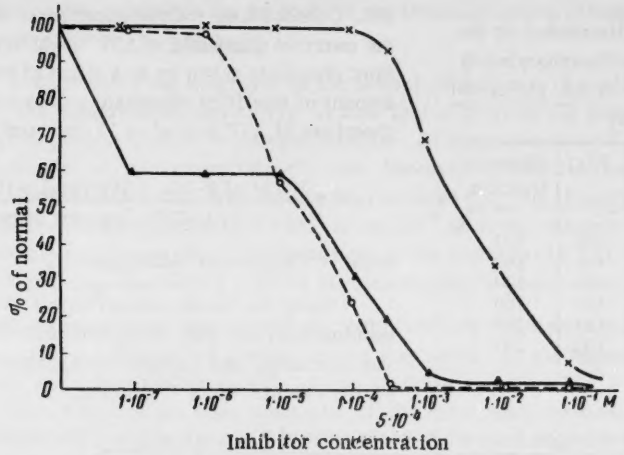

Fig. 1. The effect of sodium azide on the respiration, oxidative phosphorylation and nitrogen fixation by Azotobacter vinelandii. (In this and subsequent Figures the following legend applies x–x – respiration, o–o – P/O, ▲–▲ – nitrogen fixation)

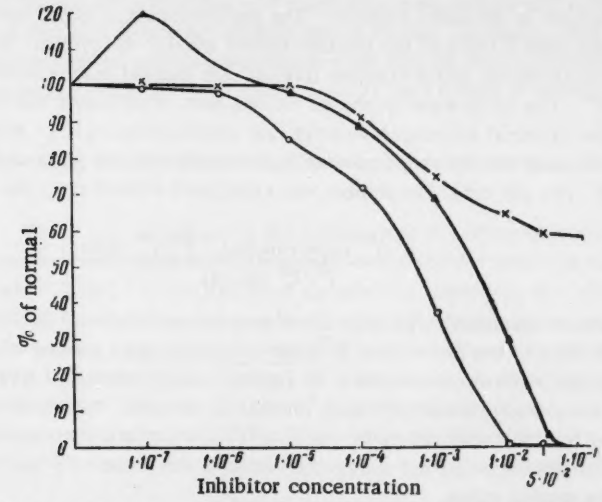

Fig. 2. The effect of potassium cyanate on the respiration, oxidative phosphorylation and nitrogen fixation in Azotobacter vinelandii.

and the mixture was shaken vigorously for 15 sec. Two ml of 10% TCA was then rapidly added and, after the content were mixed, the tubes were cooled on ice. Parallel controls were carried through with a similar quantity of killed cells. In such controls the isotope was added after the TCA. The acid-soluble phosphates were extracted twice with 5% TCA in the cold for 30 min. The nucleotides were removed from the extract by absorption onto charcoal ("Carboraffin"–made in Czechoslovakia) for one hour, with continuous stirring, in the cold. The charcoal with the absorbed nucleotides was removed by centrifugation and the supernatant was used for the estimation of the relative specific radioactivity (RSA – CPT / μatom P) of the medium inorganic phosphate. Occluded inorganic P was then removed from the charcoal by exhaustive washing (no less than 10 washes), and the completeness of washing was checked by estimating radioactivity in the washes using a Geiger-counter with a SI-2 tube. The charcoal was suspended in 2 ml of 1 N HCl and the suspension heated for 10 min at 100° to hydrolize absorbed nucleotides. The total activity of the labile P of ATP (as CPM) was estimated in the hydrolysate. As shown above, in the 15-sec experimental interval only the terminal phosphorus of ATP was labelled with P^{32}, while the

TABLE 3

The Effect of 2,4-Dinitrophenol on the Respiration, Oxidative Phosphorylation and Nitrogen Fixation in Az. vinelandii (In % of normal values)

Inhibitor conc., M.	Respiration	P/O	Nitrogen fixation
0	100	100	100
$1 \cdot 10^{-6}$	100	104	100
$1 \cdot 10^{-5}$	100	110	100
$1 \cdot 10^{-4}$	100	110	100
$1 \cdot 10^{-3}$	100	110	100
$1 \cdot 10^{-2}$	74	100	100

second phosphorus (and the terminal group of ADP) contained no label. Consequently the radioactivity of the labile P was due only to the terminal phosphate of ATP*, which originated predominantly from phosphate taken up as a result of oxidative phosphorylation. The amount of esterified phosphate was calculated as μatoms of terminal phosphate of ATP formed in 15-sec, and was given as:

$$V \times \frac{[\text{CPM of P-lab. ATP}](\text{expt}) - [\text{CPM of P-lab. ATP}](\text{contr.})}{\text{RSA (CPM}/\mu\text{atom) of medium orthophosphate}}$$

where V = volume of hydrolysate.

Using the above values (from a mean of six experimental determinations) the total phosphate esterified in one hour by 10^9 cells was calculated and expresses in μatoms. This latter value was used for the calculation of P/O ratios.

Measurement of nitrogen fixation in intact cells of Az. vinlandii, The experiments were carried out with a gaseous atmosphere containing isotopic N and O_2 in a ratio of 1:1; in order to avoid dilution with atmospheric gases, the experimental system was completely isolated. All experiments were carried out in large (100 ml) Warburg vessels in order to ensure an adequate supply of O_2 and N for the cells for one hour. Labelled N was prepared from potassium nitrate, enriched with 10% N^{15}, by decomposition with potassium hypobromite. The Warburg vessels were charged with 2 ml of the bacterial suspension (10-15 x 10^9 cells in usual medium) and with 1 ml of solution of a suitable inhibitor in the same medium. The gas mixture was passed into the vessels through the manometers for 5 min, in which time 3 liters of the mixture flowed through the system. Respiration was estimated manometrically, as described above, while nitrogen fixation was assayed mass-spectrographically as the enrichment of the cells with N^{15}. The cells were incubated for one hour, after which the nitrogen fixation was stopped by sampling one ml of the bacterial suspension directly into concentrated H_2SO_4. After wet-washing the cells with acid the ammonium salt formed was decomposed with hypobromate and the gaseous nitrogen was analyzed in the mass spectrometer MS-4. The per cent. enrichment was calculated according to the formula:

$$\frac{I^{29}/I^{28}\ \text{experimental} - I^{29}/I^{28}\ \text{natural}}{I^{29}/I^{28}\ \text{natural}}$$

Whenever the mixtures contained large amounts of nitrogenous inhibitors (DNP, NaN_3, KCNO) which could lead to a considerable dilution of the assimilated N^{15}, the suspension were washed with fresh cold medium. In each experimental series the inhibitors were tested, in duplicate experiments, at five different concentrations, and two determinations were always made with each incubation mixture. Identical experiments were repeated several times (up to five) and the mean values for each variable were later evaluated statistically. The intensity of respiration, oxidative phosphorylation and nitrogen fixation in the presence of each of the inhibitors was expressed as per cent. of the normal value.

RESULTS AND DISCUSSION

The effect of 2,4-dinitrophenol. DNP has been widely employed as an uncoupling agent of oxidative phosphorylation. In order to demonstrate its effect in the present system it was used in a range of concentrations from 10^{-6} to 10^{-2}M inclusive (Table 3).

It was found, however, that dinitrophenol had no effect on the functions investigated at any of the concentrations employed (with the exception of 10^{-2} M, which depressed repiration by 25%). It would thus appear that DPN is not a specific poison for intact Az. vinelandii as a source of nitrogen [24]. DNP was also shown to exert little or no effect on the oxidative phosphorylation in cell fragments of Az. vinelandii at concentrations 10^{-4} and

* The exchange of the type: $P^{32}O_4^{-}$ + Adenosine-P-P-P $\rightleftarrows$ PO_4^{-} + Adenosine-P-P-P^{32} cannot be entirely excluded in Azotobacter. However, over the 15-min experimental period this reaction was either insignificant or entirely absent, since its presence would lead to an observed increase in P/O ratios over increasing exposure times, while in practice the opposite was the case (cf. Table 2).

10^{-3} M [16-19]. This inhibitor is thus unsuitable for the study of the interrelationship of respiration, oxidative phosphorylation and nitrogen fixation in Az. vinelandii.

The effect of azide. Sodium azide was employed in the present experiments in a wide range of concentrations – from 10^{-7} to 10^{-1} M. The experimental results (Fig. 1) show that respiration was more resistant to the action of azide than was oxidative phosphorylation or nitrogen fixation. At concentrations 10^{-7} and 10^{-6} nitrogen fixation, but not as yet phosphorylation, was perceptibly affected. However, at azide concentrations 10^{-5} and 10^{-4}M, phosphorylation was inhibited somewhat more severly than nitrogen fixation. Finally, under conditions of complete suppression of oxidative phosphorylation by 5×10^{-4} M and 10^{-3} M azide, nitrogen fixation still proceeded and was suppressed completely only at very high concentrations of the inhibitor (10^{-2} M), when respiration was inhibited by some 60%, which indicates the lack of a direct interrelationship between the processes.

Effect of KCNO. Similar experiments were carried out with KCNO, in concentrations ranging from 10^{-7} to 5×10^{-2} M. As seen from Fig. 2, this inhibitor had a wider effect than azide. At the highest concentration employed (5×10^{-2} M) respiration was only inhibited by 40%. At the low concentration (10^{-7} M) cyanate activated nitrogen fixation and was without effect on the other functions. Of the three functions studied, oxidative phosphorylation was the most sensitive to cyanate. It was characteristic that the intensity of nitrogen fixation remained higher than that of oxidative phosphorylation over a wide range of inhibitor concentrations. An interesting phenomenon, which we have also noted in the experiments with azide, was also apparent here: with oxidative phosphorylation completely suppressed at cyanate concentrations 10^{-2} and 5×10^{-2}, nitrogen fixation continued to take place, which indicates the independence of the two processes.

It was shown previously by one of us [21] that ATP synthesis ceased completely in Az. vinelandii cells exposed to 5×10^{-4} M azide and that ATP levels fell sharply after a 30-min incubation with the inhibitor. The continuing nitrogen fixation observed in the present experiments even after an hour's exposure to considerably higher concentrations of azide and cyanate could not have taken place at the expense of stored endogenous high-energy phosphates of the cell.

It may therefore be concluded that the energy of oxidative phosphorylation is not essential for the process of nitrogen fixation.

The effect of X-rays. There are indications in the literature to the effect that ionizing radiation, and particularly X-rays, uncoupled oxidative phosphorylation while being without effect on either respiration or nitrogen fixation in microorganisms [25, 26]. X-irradiation of Azotobacter suspension thus offered another means of checking our hypothesis about the lack of direct connection between the energy of oxidative phosphorylation and nitrogen fixation. Forty-hour cultures of Azotobacter vinelandii were irradiated with X-rays in doses of 2, 10, 25 and 40 Kr respectively, after which they were "grown" for a further period of 22 hours in normal medium in order to allow the cells to emerge from the state of "shock" which invariably follows the irradiation of Azotobacter cultures with X-rays. As can be seen from Table 4, oxidative phosphorylation was found to be the most radiosensitive, and nitrogen fixation and respiration the least sensitive, of the processes investigated.

TABLE 4

The Effect of X-Irradiation on the Respiration, Oxidative Phosphorylation and Nitrogen Fixation in Az. vinelandii (in % of normal values)

Irradiation dose, Kr.	Respiration	P/O	Nitrogen fixation
0	100	100	100
2	111	80	126
10	91	60	100
25	74	20	40
40	55	0	5

It is of interest to note again the lack of any interdependence between the processes of oxidative phsophorylation and nitrogen fixation: under the conditions of severe suppression of the former process after irradiation with 2 and 10 Kr, the latter process was not affected, and was actually somewhat activated at the 2 Kr does level.

The energy of oxidative phosphorylation is known to be essential in the processes of protein and nucleic acid synthesis, with the participation of ammonia produced by azotobacter in the process of nitrogen fixation. It may therefore be assumed that, indirectly at least, oxidative phosphorylation should influence nitrogen fixation. In order to verify this assumption experimentally, the intensity of respiration, oxidative phosphorylation, nitrogen fixation and ammonia assimilation by Azotobacter vinelandii were compared in cultures grown on either molecular or ammonia nitrogen, and in the presence of a range of concentrations of azide.

TABLE 5

The Inhibition by Sodium Azide of Respiration, Oxidative Phosphorylation, and Fixation of N^{15} From Molecular or Ammonia Nitrogen in Az. vinelandii

Expt. No.	Inhibitor Concentration, M	Respiration (O_2 uptake)				P/O		N^{15} incorporation	
		on N_2	on NH_4^+	on N_2	on NH_4^+	on N_2	on NH_4^+	N_2 fixation	NH_4^+ assimilation
		μ atoms/hr/10^9 cells		per cent. of normal					
1	0	1,42	1,97	100	100	100	100	100	100
2	$1 \cdot 10^{-5}$	1,42	2,42	100	123	58	100	60	100
3	$1 \cdot 10^{-4}$	1,42	2,42	100	123	26	60	32	100
4	$5 \cdot 10^{-4}$	1,35	—	95	—	0	—	20	—
5	$1 \cdot 10^{-3}$	1,00	1,80	70	90	0	20	5	33
6	$1 \cdot 10^{-2}$	0,40	0,60	28	32	0	0	0	0
7	$1 \cdot 10^{-1}$	0	0,15	0	8	0	0	0	0

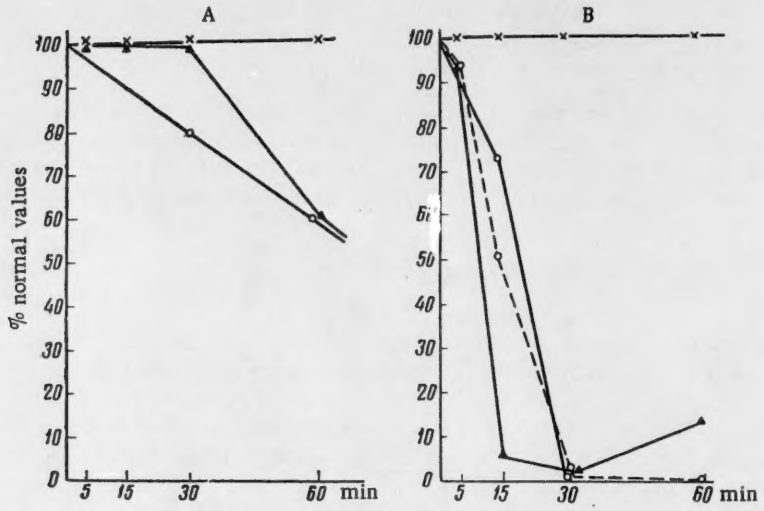

Fig. 3. The time-progress of respiration, oxidative phosphorylation and nitrogen fixation in the presence of 10^{-5} M (A) or 5×10^{-4} M (B) azide. (Dotted lines: ATP. Others, as in Fig. 1)

A comparative study of the three processes in Az. vinelandii actively fixing nitrogen and with nitrogen fixative suppressed. In order to obtain comparable values, the two cultures of Az. vinelandii were both harvested in their respective logarithmic phases of growth, which corresponded to 24 hours for the nitrogen-fixing culture and 20 hours for the culture grown on ammonium salts. In the case of suspensions grown on ammonium salts, the incubation medium contained labelled N in the form of $(N^{15}H_4)_2SO_4$ in concentrations 0.2 mg of the salt per ml, while the gas phase consisted of a mixture of equal volumes of O_2 and "cold" N_2. Control incubations with "cold" $(NH_4)_2SO_4$ in the incubation mixture and with labelled N^{15} in the gaseous phase showed that, at the concentrations of ammonium salt used in the medium, the uptake of gaseous nitrogen was practically completely suppressed.

The results dealing with the effect of NaN_3 on the processes of respiration, oxidative phosphorylation, nitrogen fixation and NH_4^+ assimilation in Az. vinelandii are shown in Table 5.

It will be seen from Table 5 that the processes of respiration and of oxidative phosphorylation proceed far more intensively in the presence of ammonia nitrogen than on molecular nitrogen, so that considerably higher concentrations of the inhibitor were required in the former case in order to attain the same degree of inhibition. It is of interest to note that under suppression of oxidative phosphorylation the assimilation of ammonia nitrogen was completely abolished (Table 5, expts. 6 and 7, NH_4^+ as N source), while nitrogen fixation continued (ibid,

expts 4 and 5, N_2 as N source). This indicates that the process of ammonia assimilation is much more directly dependent on oxidative phosphorylation than is nitrogen fixation. The results tempt the suggestion that the dependence of nitrogen fixation on oxidative phosphorylation is not direct, but is mediated through nitrogen utilization in the synthesis of organic nitrogenous compounds, mainly proteins.

It may be suggested that the interference with protein synthesis in Azotobacter cells leads to a kind of "self-inhibition" of nitrogen fixation, presumably caused by product inhibition as various intermediates accumulate and exercise a certain amount of feedback. In order to verify this suggestion experimentally we have tested the effect of chloramphenicol, well established inhibitor of protein synthesis, on the process of nitrogen fixation.

The effect of chloramphenicol. The cultures of Az. vinelandii were grown for 24 hours in nitrogen-free media, from which they were transferred for a further 12 hours into fresh media containing chloramphenicol in the following range of concentrations: 10^{-5}, 10^{-4}, $5 \cdot 10^{-4}$ and 10^{-3} M.

In this experimental series, apart from respiration, oxidative phosphorylation and nitrogen fixation, we have also measured N_2^{15} incorporation into protein. For this purpose the cells, after the usual incubation period, were washed firstly with cold 5% TCA, then twice extracted for 15 min with hot TCA (90°) in order to remove nucleic acids and other non-protein nitrogenous matter. The results of these experiments (Table 6) showed that chloramphenicol was almost without effect on either respiration or oxidative phosphorylation (some effect was observed only when CAP concentration reached 10^{-3} M). However, already at 10^{-4} M, chloramphenicol depresses protein synthesis* 50% and, apparently, indirectly affected nitrogen fixation. Raising the inhibitor concentration to 5×10^{-4} M and 10^{-3} M completely abolished protein synthesis which in turn suppressed nitrogen fixation to one-third of normal level.

TABLE 6

Effect of chloramphenical on the respiration oxidative phosphorylation and nitrogen fixation of Az. vinlandii (in % of normal values)

Concentration of chloramphenical, M	Respiration	P/O	Nitrogen fixation	
			normal	incorporation of N^{15} in protein
0	100	100	100	100
$1 \cdot 10^{-5}$	100	100	100	80
$1 \cdot 10^{-4}$	100	100	62	50
$5 \cdot 10^{-4}$	90	75	34	0
$1 \cdot 10^{-3}$	80	46	28	0

Thus, in the cells of Az. vinelandii the inhibition of protein synthesis caused an accumulation of the products of nitrogen fixation, which suppressed the latter process by some mechanism of product inhibition.

A kinetic study of the processes of respiration, oxidative phosphorylation and nitrogen fixation. In order to gain a better understanding of the interrelationship of the three processes under investigation, a kinetic study was made of the inhibition by NaN_3 of these processes in Az. vinelandii over time intervals of 5, 15, 30 and 60 min after exposure to the inhibitor. Two concentrations of azide 10^{-5} M and 5×10^{-4} M, were used. As shown above, one hour's exposure to 10^{-5} M NaN_3 depressed both oxidative phosphorylation and nitrogen fixation by 40%; at 5×10^{-4} M azide oxidative phosphorylation was completely abolished, while nitrogen fixation continued at 20% of normal levels. In both cases respiration remained normal (Fig. 1).

The results of the kinetic experiments are presented in Figs. 3A and 3B. It will be seen from the Figures that with 10^{-5} M azide, oxidative phosphorylation was severely inhibited in the first 30 min, while neither respiration nor nitrogen fixation were affected. The complete lack of direct interdependence between nitrogen fixation and oxidative phosphorylation was particularly clearly demonstrated in this initial incubation period. Continued exposure to the inhibitor (for 30 and 60 min) brought about severe inhibition of both processes; it remained obvious, however, that nitrogen fixation was less severely affected than oxidative phosphorylation.

An entirely different picture is presented by the experiment with sodium azide present at 5×10^{-4} M. Here the effect of the inhibitor on nitrogen fixation was already apparent at 15 min, and the process was severely depressed after 30 min.

In the time interval that followed (30-60 min) a slight recovery of the nitrogen fixation was observed. The process of oxidative phosphorylation, while relatively less affected than nitrogen fixation during the initial few minutes of incubation, ceased completely after 30 min. In the subsequent time interval we have again encount-

* The rate of N^{15} incorporation into protein can serve, to a certain extent, as an indication of the rate of protein synthesis.

ered the phenomenon previously observed with the various inhibitors and with X-irradiation, namely the continuation at a low level, of nitrogen fixation on the background of complete suppression of oxidative phosphorylation. In the presence of 5×10^{-4} M azide the ATP content of the cells fell proportionally with the decrease in oxidative phosphorylation and, towards the end of the first 30-min period, the cellular ATP was nearly depleted(Fig. 3B). This again indicates that nitrogen fixation took place without the utilization of high-energy phosphorus compounds.

The results thus appear to verify the theory that the high-energy phosphates in the cell are not a source of energy for nitrogen fixation. The process of nitrogen fixation is, apparently, also independent of respiration since, in the presence of a number of inhibitors, conditions could be be created where nitrogen fixation was depressed, while respiration remained normal. The possibility is not excluded that nitrogen fixation is specifically more sensitive to the inhibitors used than is respiration. It should also be remembered that some existing anaerobic bacteria are capable of nitrogen fixation in the absence of any respiration [27, 28].

SUMMARY

Respiration, oxidative phosphorylation and nitrogen fixation in Azotobacter vinelandii were not affected when intact cells were exposed to 10^{-6} to 10^{-3} M 2,4-dinitrophenol.

In the presence of sodium azide (10^{-7} to 10^{-1} M) or potassium cyanate (10^{-7} to 10^{-2} M), at limiting concentrations of each which inhibited oxidative phosphorylation, the two drugs showed a common tendency of effect: with complete inhibition of oxidative phosphorylation there still remained a considerable level of nitrogen fixation. These observation are taken as an indication of the absence of direct connection between the two processes.

X-irradiation was found to affect most severly oxidative phosphorylation, while nitrogen fixation was considerably less inhibited, and respiration remained unaffected. Here again under certain conditions a high level of nitrogen fixation persisted while oxidative phosphorylation was suppressed.

In the presence of chloramphenicol ($10^{-5} - 10^{-3}$ M) respiration remained almost unaffected, while oxidative phosphorylation was only slightly inhibited. When present in concentrations 5×10^{-4} and 10^{-3} M, the latter antibiotic completely suppressed the incorporation of $N^{15}H_4^+$ into protein. With protein synthesis at a standstill, various nitrogenous intermediates accumulated and their "back pressure" resulted in product inhibition of nitrogen fixation.

Investigation of the time-course of inhibition of respiration, oxidative phosphorylation and nitrogen fixation in the presence of inhibitors also demonstrated the lack of direct connection between the three process investigated in the intact Azotobacter.

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TRANSFER OF GLUCOSYL RESIDUES OF MALTOSE IN SKELETAL MUSCLE

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This is a report of results obtained in a study of optimal conditions for the action of a transglycosylase which catalyzed the transfer of glucose residues of maltose—an enzyme shown to be present in muscle [1]. Enzyme activity was studied in relation to incubation temperature, pH, and substrate concentration.

METHODS

Source of enzyme was autolyzed and dialyzed muscle extract [1]. In contrast to previous experiments, however, dialysis was carried out for a longer period (1 day) to ensure complete removal of oligosaccharides. Experiments were performed at high and low levels of maltose, which was used both as a commercial preparation and as chromatographically purified samples.

The reaction mixture was analyzed by descending paper chromatography. A 3:2:1 butanol—ethanol—water mixture was used as a solvent system, and papers were sprayed with aniline phthalate. Separation was carried out on No. 2 rapid Leningrad paper. Markers employed were: glucose, Rf 0.28 (determined after a 19 hour resolution period, Fig. 1, d); maltose, Rf 0.129 (calculated from glucose R after three day resolution period, Fig. 1, a); maltotriose, which was isolated by chromatographing commercial maltose preparations and eluting strips corresponding to trisaccharide with 50% ethanol. This preparation was rechromatographed and analyzed. Analysis revealed that it was chromatographically homogeneous, and that its position corresponded to that of the contaminant saccharide in the maltose preparation (Fig. 1, b).

The ratio of reducing group values (determined according to Somogyi [2]) before and after acid hydrolysis is 3:1, indicating that the substance in question is a trisaccharide. Treatment of the preparation with salivary amylase and maltase results in glucose formation (Fig. 1c). This indicates that only α-1,4-glucosidic linkages are present, i.e., identifies the substance as maltotriose.

TABLE 1

Hydrolysis of Maltose by Muscle Extracts. Reducing capacity, in mg glucose per ml; pH 4.8; incubation at 37°

Expt. no.	Maltose conc. %	Incubation period			
		0	4 hr.	1 day	3 days
1	0,01	0,176	0,270	0,432	0,358
	0,1	0,956	**0.196**	1,656	1,704
2	0,01	0,190	**0.196**	0,282	0,310
	0,1	1,008	1,096	1,366	1,412

The Rf of the isolated trisaccharide, calculated from the Rf of glucose was 0.054. The ratio of maltose Rf to maltotriose Rf, 2.4 according to our data, is in agreement with that calculated from the data of other workers [3]. The amount of maltotriose in separate spots on the chromatogram, according to analysis, was 60 μg in one spot (a) and 1000 μg in the other (b).

In experiments with small amounts of maltose, the increase in reducing capacity was measured (Hagedorn-Jensen method). The reaction mixture was incubated for 2-3 days.

RESULTS

Rate and character of reaction in relation to substrate concentration. Experiments with a graded series of maltose concentrations revealed that not only the rate but the character of the reaction depends on substrate concentration. At low concentration (0.01-0.1% maltose) only a hydrolyzing action was apparent; this was designated

TABLE 2

Effect of Incubation Temperature on Decomposition of Maltose by Muscle Extracts. Reducing capacity in mg glucose per ml

Incubation mixture	37°			50°			60°			70°			80°			90°			Boiled extract (incubation at 37°)		
	0	3 hr	1 day	0	3 hr	1 day	0	3 hr	1 day	0	3 hr	1 day	0	3 hr	1 day	0	3 hr	1 day	0	3 hr	1 day
4.5 ml extract, 0.3 ml 2.5% maltose, 0.5 ml 1M acetate buffer, pH 4.8	1,025	1,205	1,480	1,005	1,415	1,635	1,056	1,300	1,415	0,945	1,285	1,305	0,955	0,990	—	1,00	1,060	1,075	0,915	0,915	0,915
4.5 ml with water, 0.3 ml 2.5% maltose, 0.5 ml 1M acetate buffer, pH 4.8	0,930	—	0,930	0,930	—	0,930	0,885	—	0,885	0,805	—	0,805	—	—	—	0,890	—	0,915	0,930	—	0,930

the maltase effect. These experiments were characterized by an increase in reducing capacity of the of the incubation mixture (Table 1) and by the appearance of a fairly large glucose spot on chromatograms. The formation of maltotriose did not take place (Fig. 2); the barely visible trisaccharide spot showed no change even after prolonged incubation. At high substrate concentrations another pattern emerged (Fig. 3). Under these conditions, a maltotriose spot was always found together with the glucose spot after incubation. Maltose in concentrations from 0.75 to 32% were investigated.

Fig. 1

Fig. 2

Fig. 1. Chromatographic investigation of marker preparations. a-mixture of markers (I–maltotriose, II–maltose, III–glucose); b- maltotriose; d- glucose; c- maltotriose treated with amylase and maltase; F-solvent front.

Fig. 2. Chromatographic study of the effect of extract in 0.01-0.1% maltose solution. Here and in Fig. 3-5: 1–markers; 2–reaction mixture before incubation; 3, 4, 5–the same, 1, 2, and 3 days after incubation. I–maltotriose; II–maltose; III–glucose.

These experiments showed that the largest amount of trisaccharide and glucose was formed at a 1-7% maltose concentration. At higher concentrations, the maltase effect of the enzyme was depressed, and at concentrations of 20-30% it was almost absent.

Since optimal enzyme activity occurred at 1-7% maltose, we utilized a concentration of 7% in all succeeding experiments, which ensured the most favorable conditions for chromatographic investigations.

Determination of optimal pH. Studies of enzyme activity at various pH's (3.8, 4.8-5.0; 7.0; 8.5-9.0) showed that the optimal pH is

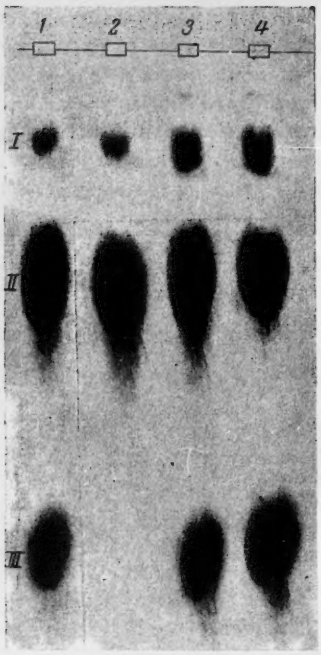

Fig. 3

Fig. 4

Fig. 3. Chromatographic study of the effect of extract in 7% maltose.

Fig. 4. Chromatographic study of the effect of extract on maltose at pH 4.8.

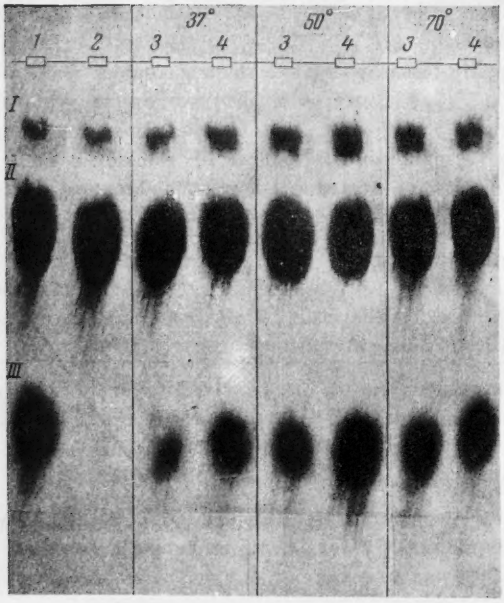

Fig. 5. Chromatographic study of the effect of extract on maltose at various temperatures.

4.8-5.0. Under these conditions (Fig. 4), both glucose and maltotriose formation were at a maximum.

It should be noted that at pH 7.0 there was also vigorous formation of glucose, but the maltotriose spot was smaller than at pH 4.8-5.0. Under these conditions new spots lying next to the trisaccharide spot above and below it were formed. Judging by their positions, they belonged to the isomaltose series. Their presence on the chromatograms indicated that the muscle preparations contained enzymes mediating their formation.

Determination of temperature optimum of the reaction. Experiments were performed at various incubation temperatures using low and high maltose concentrations. Activity was measured over a temperature range of 8 to 90 deg. It was found that the optimal temperature is 50 deg.

With low maltose concentrations, glucose accumulation was greatest at an incubation temperature of 50 deg, whether incubation periods were 3 hours or 1 day (Table 2). Enzyme activity is markedly lower at 80 and 90 deg.

With high maltose concentrations, the temperature optimum was also 50 deg. At this temperature

maltotriose formation was greatest (Fig. 5).

Thus, at low concentrations, where only the hydrolyzing action of maltase occurs, and a high concentrations, which are associated with accumulation of trisaccharide, the optimal temperature was 50 deg.

DISCUSSION

These experiments have shown that transglycosylase of autolyzed and dialyzed muscle extracts, which catalyzes transfer of glucosyl residues of maltose with formation of maltotriose, has the same pH optimum, in the acid zone, as a similar enzyme from liver [4]. However, muscle transglycosylase possesses considerable thermostability (temperature optimum 50 deg), and is quite active in the presence of large amounts of substrate.

At low substrate concentrations, there is only a maltase action. In this case, the hydrolytic action is regarded as a special case of the transfer reaction, the glucosyl residue being transferred to water.

At high substrate concentrations, hydrolysis is accompanied by trisaccharide synthesis. There is evidence that the enzyme in question is able to transfer glucosyl residues to a saccharide acceptor. The fact that both enzymatic activities have the same temperature optimum (50 deg) indicates a similarity of the enzymes involved. Apparently both the hydrolyzing and synthesizing actions are mediated by the same enzyme, muscle maltase, which was previously thought to have only hydrolyzing activity.

SUMMARY

A transglucosylase from skeletal muscle type, which catalyses the transfer of glucosyl residues of maltose with the formation of glucose and of maltotriose was studied.

It was shown that its maximum activity occurs at pH 4.8-5.0, 50°, and 1–7% substrate concentration. Incubation of the extract with small amounts of maltose (0.01-0.1%) results in the formation of glucose only, i.e. only the maltase effect is apparent. With greater amounts of maltose (1–7%), accumulation of both glucose and maltotriose is observed. Both effects are maximal at the same temperature (50°), which suggests that the same enzyme catalyses maltose breakdown and transfer of glucosyl residues of maltose to an acceptor, thus forming a trisaccharide. Muscle maltase, to which only a hydrolytic effect has thus far been attributed is presumably able to transfer the glucosyl residues of maltose to a saccharide acceptor and to synthesize a trisaccharide.

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SOME PECULIARITIES OF MITOCHONDRIA FROM TISSUE CULTURE CELLS

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It is well known that tissue explants resemble malignant tissues in the character of their metabolism [1-3]. It is commonly assumed [4-7] that mitochondria from malignant tumors differ with those from normal tissues in several ways – in low oxidative activity, in high ATPase and diphosphopyridine nucleotidase activity, in comparative stability to the action of certain swelling agents, etc. Determination of the extent to which these features are characteristic of mitochondria from cells of tissue explants is of value from the point of view of establishing general patterns of mitochondrial structure and function in cells not subject to regulation by the organism.

In a previous report [8], certain characteristics of mitochondria from a strain of cells originating from monkey heart (MH) were described. In continuation of these studies, material on the ATPase activity of mitochondria from primary tissue explants and from continuous strains, as well as their swelling behavior and their response to certain factors, is reported here.

METHODS

Biological material used was: primary cultures of cortical tissue of monkey kidney (MK), fibroblasts of 9-11-day-old chick embryos (FCE), and continuous strains of MH and HEP-2. Culture conditions and methods of separating cells and isolating mitochondria have been described [1, 8]. Cell fractionations were carried out in various solutions: in 0.25M sucrose; in the same solution prepared in 0.01M tris buffer, pH 7.2, the latter containing in some cases 0.001M EDTA; in 0.15M KCl; in a mixture recommended for isolation of mitochondria from muscle [9]. In a number of cases, mitochondria were isolated from the liver of healthy white mice by the usual method [10]. These were used as a standard of comparison, being the most thoroughly studied.

Mitochondrial swelling was measured in an SF-4 spectrophotometer by Cleland's method [11] according to optical density of solutions at 520 mμ; either 0.25M sucrose or 0.15M KCl with trisbuffer, pH 7.4, was used as a suspension medium; the amount of nitrogen per sample of mitochondria was about 0.1-0.15 mg. ATPase activity was determined in 0.0067M tris buffer, pH 7.4, at 37 deg for a period of 15 minutes. Phosphorus was determined by the method of Fiske and Subbarow [12] or by that of Lowry and Lopez [13]. Activity was expressed in μmoles released $P_{inorg.}$/mg N, which later was detemined colorimetrically with Nessler's reagent.

The following preparations were employed: thyroxin from Hungary, crystalline trypsin from the Institute of Biochemistry, Academy of Medical Sciences, USSR, Na deoxycholate (DOC) from Eastman Kodak Corp., EDTA from Nutritional Biochemicals (USA), ATP from the same source and from the Ivanov Meat Combine.

RESULTS

Optical density data on spontaneous mitochondrial swelling are presented in Table 1. It is apparent that mitochondria from tissue explants are, in general, relative stabile. After 45 minutes, the average change in optical density was ~10%, irrespective of the fractionation medium or suspension medium. In certain cases, however,

TABLE 1

Swelling of Mitochondria from Various Tissue Cultures

Tissue culture	Fractionation medium	Suspension medium	Fall in optical density, %		Tissue culture	Fractionation medium	Suspension medium	Fall in optical density, %	
			10 min	45 min				10 min	45 min
MH	stb	Sucrose	1,0	6,5	MK	stb	Sucrose	0	5,0
	»	»	—	3,5		»	»	8,0	19,0
	»	»	2,0	7,5		»	»	0,5	3,5
	»	»	1,5	11,0		»	»	8,5	19,0
	»	»	—	10,0		st	KCl	6,5	14,0
HEP-2	stb	Sucrose	4,0	7,0		»	»	4,0	8,0
	»	»	4,0	7,5		»	»	5,5	14,5
	»	»	3,0	10,0	FCE	stb	Sucrose	0,5	4,0
	Sucrose	»	3,5	8,5		»	»	1,0	6,0
	»	»	3,5	10,0		medium	KCl	1,5	3,0
	»	KCl	3,0	12,0		st	»	2,0	6,5
	»	Sucrose 0,88 *M*	4,5	9,0		»	»	4,0	10,0
						»	»	5,5	12,5

Legend. stb – sucrose + 0.02 M tris buffer + 0.001 M EDTA, pH 7.4; st – sucrose + 0.02 M tris buffer, pH 7.4; KCl – KCl + 0.02 M tris buffer, pH 7.4

for example with MK mitochondria, change in optical density was as high as 20%, even though experimental conditions (culture age, isolation method, etc.) were duplicated as far as possible. The reason for these variations is not clear and is probably related to experimental details which have as yet been neglected.

Addition of EDTA directly to the suspension medium was generally without effect on mitochondrial swelling (Fig. 1). As a rule, ATP was also without effect (Fig. 2), although there were certain rare exceptions, such as the tendency of FCE mitchondria to show a reversal of swelling in its presence (April 26 expt.). Mitchondria from tissue cultures, in contrast to those from liver, were completely resistent to thyroxin (Fig. 3).

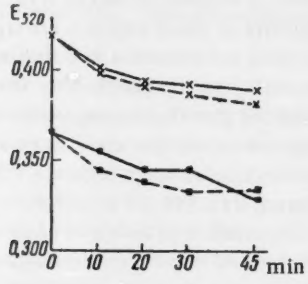

Fig. 1. Effect of EDTA on swelling of mitochondria from tissue culture cells. Suspension medium – 0.25M sucrose in 0.02M tris buffer, pH 7.4. Final concentration of EDTA, $5 \cdot 10^{-4}$M. In this and succeeding figures, crosses – MK mitochondria, squares – HEP-2 mitochondria. Solid lines represent spontaneous swelling, dotted lines – change in optical densities of mitochondrial suspensions in the presence of EDTA.

It is well known that the mitochondrial membrane is made up mainly of lipoprotein complexes and is sensitive to agents which destroy lipids and proteins [7, 14, 15]. Trypsin acts on proteins, while DOC acts primarily on lipids; the latter is widely used to disrupt mitochondria when it is desired to isolate sub-mitochondrial particles.

Treatment of mitochondria from tissue cultures with trypsin results in a rapid and pronounced fall in optical density (Fig. 4). Ordinarily the action of this enzyme is completed in 15-20 minutes, and subsequently the optical density curves for control and experimental samples are parallel. Under these same conditions, liver mitochondria prove resistant to trypsin, judging by the optical density change (Fig. 4). An increase in trypsin concentration does not alter its effect. A trypsin inhibitor completely suppresses its action.

The swelling behavior in the presence of DOC is almost opposite in character. With addition of this compound to a suspension of liver mitochondria, the optical density exhibits an immediate pronounced change. At $0.9 \cdot 10^{-3}$M DOC, optical density falls 40-50% in 20 minutes. In suspensions of HEP-2, MK, and FCE mitochondria containing approximately the same amount of nitrogen, optical density falls only 15-20%. If DOC concentration is increased to $9 \cdot 10^{-3}$M, optical density of suspensions of such mitochondria begins to fall sharply, so that the difference between them and liver mitochondria is beginning to disappear (Table 2). Thus, the lipid components of the membrane of tissue culture mitochondria prove to be less sensitive to DOC than do those of liver mitochondria membranes, and higher concentrations of DOC are required to disrupt the former.

Data on ATPase activity of homogenates and mitochondria of tissue explants, and also on the effect of di-

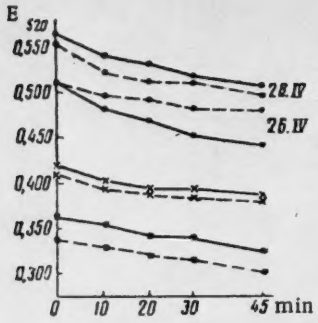

Fig. 2. Effect of ATP on swelling of mitochondria from tissue culture cells. Final concentration of ATP, $1 \cdot 10^{-3}$ M. Solid circles – mitochondria from FCE.

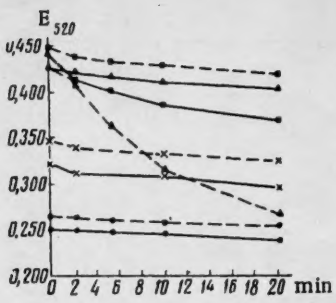

Fig. 3. Effect of thyroxin on swelling of various mitochondria. Final concentraion of thyroxin, $5 \cdot 10^{-3}$ M. Triangles – mouse liver mitochondria.

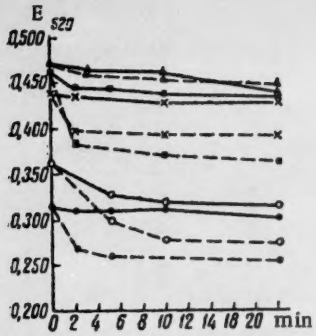

Fig. 4. Effect of trypsin on optical density change in suspensions of various mitochondria. Final concentration of trypsin, 50 μg/ml. Open circles – MH mitochondria.

TABLE 2

Effect of Various Concentrations of DOC Change in Optical Density of Suspensions of Mitochondria from Different Sources

Mitochondria	$0{,}09 \cdot 10^{-3}$ M		$0{,}9 \cdot 10^{-3}$ M		$9 \cdot 10^{-3}$ M	
	fall in E, %, in time designated, min.					
	10	20	10	20	10	20
Liver	6	6	46	42	58	—
MK	3	2	17	16	42	42
FCE	—	0	—	21	—	44
HEP-2	4	—	15	19	32	36

Legend. All samples contain approximately the same amount of mitochondrial nitrogen.

nitrophenol (DNP) and Mg^{++} on this enzyme, are presented in Table 3. It is evident that FCE homogenates and mitochondria have the highest activity. In contrast to the other tissue explants, ATPase of this tissue is activated by DNP, especially in the homogenates. The enzyme is activated quite strongly by Mg^{++} in all cases.

DISCUSSION

Examination of the results obtained makes it clear that in a number of respects (effect of ATP, EDTA, thyroxin) mitochondria of tissue explants are significantly different from liver mitochondria and similar to mitochondria from malignant tissues [7, 17]. It should, however, be pointed out that these comparisons are somewhat arbitrary, inasmuch as a single agent, such as thyroxin, often affects mitochondria from different malignant neoplasms in different ways [16, 17]. Moreover, mitochondria from various healthy tissues also differ in some respects [18, 19]. The method of isolation of mitchondria is of considerable significance; the problem of the more labile character of mitochondria from malignant tissues has been repeatedly discussed [20, 21]. It should also be noted that mitochondria from tissue cultures are heterogeneous in character, as has been observed by morphologists [22, 23]. These reservations do not rule out the fact, however, that the aggregate mitochondria of tissues explants are distinguished by a number of features believed to be characteristic of tissues degenerating to the malignant condition. Evidently the definite metabolic pattern characteristic of cells freed from regulatory control and capable of unlimited growth without differentiation is conditioned by the properties of the cell organelles, mitochondria in particular.

Some conception of these properties may be arrived at from experiments with trypsin and DOC. Liver mitochondria are more sensitive to DOC and more resistant to trypsin than mitochondria from tissue explants. These data require more intensive analysis, however, since they are concerned only with changes in optical density of mitochondrial suspension. Already, though, the impression arises that protein and lipid components of tissue explant mitochondria differ from those of mitochondria from normal tissues (in this case liver)either in their disposition, their amount, or their structure. It might be supposed that the ratio of mitochondrial lipid to protein varies from tissue to tissue. According to Mutolo [7], mitochondria from a number of malignant and normal tissues also respond differently to DOC and trypsin and exhibit different pemeability properties [24]. Here it is relevant to mention morphological studies in which it has been shown that mitochondria of tissues degenerating to a malignant condition are incompletely formed [25]. It is interesting to note the association in mitochondria from tissue

TABLE 3

Adenosine Triphosphatase Activity (μmoles P/mg N) of Homogenates and Mitchondria from Tissue Explants. Incubation at 37°, 15 min. N content of a sample, 0.15-0.3 mg, DNP—$1 \cdot 10^{-3}$ M; Mg^{++}—$2.5 \cdot 10^{-3}$M

Tissue culture	Homogenate			Mitochondria		
	no additive	DNP	Mg^{++}	no additive	DNP	Mg^{++}
MK	1,0	1,3	—	2,5*	—	—
	2,2	2,2	—	2,4	2,4	—
	1,4	1,4	4,8	1,9	1,9	4,6
	—	—	—	1,6	1,6	4,3
HEP-2	1,1	0,9	2,9	2,0	—	3,3
	1,5	—	3,7	1,0	0,9	—
	1,3	1,3	3,3	2,3	1,9	—
	—	—	—	2,3	2,3	2,8
FCE	3,6	7,1	8,4	3,7	—	—
	2,7	4,8	8,9	6,1	8,1	16,5
	—	—	—	4,3	—	—
	3,0	3,5	—	5,0	5,0	—

*Figures were obtained in different experiments and are therefore not comparable.

explants and malignant tissues of relatively high resistance to a "lipid agent"—DOC with a defective oxidative chain [1, 26]; it is well known that lipid components are particularly significant in electron transport [27]. Thus, selective fragmentation of the mitochondrion may prove a convenient means of elucidating the properties of mitochondria from various sources.

The data on ATPase activity must be assessed with some care. Thus, in connection with the effect of DNP on ATPase activity in hepatoma mitochondria, it is known that with different methods of isolation the effect of DNP is different [20]. Data on comparative activities for mitochondria of different tissues are also contradictory [20, 22].

In our experiments it may be considered firmly established that homogenates and mitochondria of the tissue explants studied possess a clearly defined ATPase activity. It should be noted that ATPase activity of whole cells, particularly of HEP-2 cells, is extremely low, according to preliminary data. This is an agreement with published findings [29]. The enzyme present in homogenates and mitochondria of tissue cultures is undoubtedly activated by Mg^{++}, and apparently that in MK and HEP-2 cells is not activated by DNP. During "ageing", enzyme activity does not, as a rule, increase. These indications of "organization" of ATPase activity in mitochondria from tissue cultures, namely from MK and HEP-2 cultures, are more reminiscent of mitochondria from malignant tissues than from normal tissues.

ATPase of FCE is uniquely distinguished from the other ATPase's. On a nitrogen basis, enzymatic activity is higher than in other cultures. DNP activates ATPase activity of homogenates and sometimes of mitochondria from these cells. Whether these differences are related to the fact that FCE cultures originate from embryonic cells, or whether they are determined by inherent peculiarities of FCE tissue is not yet clear. Data on the difference between mitochondria of primary, "unstabilized" cultures and mitochondria of continuous cultures are meager. Further study at the "mitochondrial" level would probably yield the clearest explanation of the metabolic peculiarities of various tissue explants, which are special examples of the type of tissue whose functional activity resides wholly in unlimited growth.

SUMMARY

Swelling of mitochondria from various tissue explants (monkey kidney cortex tissue, fibroblasts of a 9-11--day-old chick embryo, a cancer strain HEP-2) is unaffected by $3 \cdot 10^{-5}$ thyroxine.

Agents which act on proteins and lipids (trypsin and deoxycholate) affect mitochondria from tissue explants and those from liver differently. In the presence of trypsin, suspensions of tissue explant mitochondria, in contrast to liver mitochondria suspensions, exhibit a rapid and pronounced fall in optical density. In the presence of $0.9 \cdot 10^{-3}$

M deoxycholate, however, optical density of liver mitochondria suspension undegoes an immediate marked change while mitochondria of tissue explants prove to be considerably more resistant to this agent.

Homogenates and mitochondria from the tissue explants investigated possess a clearly discernible ATPase activity. Preparations from fibroblasts have the highest activity (4-5 μmoles P per mg N). The enzyme is strongly activated by Mg++ in every case.

The authors wish to express their gratitude to their colleagues of the encephalitis laboratory for the gift of the fibroblast culture.

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A PAPER CHROMATOGRAPHIC STUDY OF CARBOHYDRATES USING A DENSITOMETER

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Carbohydrates present in the tea leaf and their fate during the processing of tea have been little studied. The only published reports are concerned with amounts found in the plant [1-6]. In view of recent findings of the interaction of carbohydrates with amino acids, peptides, and proteins at high temperatures, carbohydrates, particularly monosaccharides, must contribute to the aromatic qualities of many foodstuffs, including tea [7, 8].

Existing methods of sugar determination as applied to tea are complex and tedious [9]. This study is devoted to the carbohydrate composition of the tea leaf and of processed tea as determined by paper chromatography. After spraying of the chromatograms to render the spots visible, quantitative measurement of amounts of carbohydrate on the paper was made by the use of a photoelectric densitometer of the Shipalov type [10], which rapidly scans the paper.

METHODS

Green tea leaves fixed in steam were analyzed. Since tea contains resins, pigments, and tannins which interfere with sugar determination, the material was first treated with dry chloroform. Six grams of ground and sifted material was enclosed in a packet of filter paper and extracted with dry chloroform in a Soxhlet apparatus. After a 10-15-hour extraction, remaining traces of chloroform were removed by suction.

5-g sample of chloroform-treated air-dry material was then extracted 6 times with five volumes of 95% alcohol on a water bath at 70 deg, using a reflux condensor [11]. Each extraction continued for 30 minutes. The alcohol extract was concentrated in vacuum at 40 deg on a water bath until all the alcohol had distilled off. The flask's contents were then thoroughly washed several times with warm water to extract sugars, and the wash water was quantitatively transferred to a 25-ml volumetric flask and left over night in the refrigerator to permit precipitation of tannic substances. The clear solution was spotted with a 0.05-ml micropipette onto strips of slow Leningrad paper 250 x 30 mm with a neck 10 x 3 mm [12]. A 40:11:19 n-butanol–ethanol–water mixture was employed as the solvent system. Development of the chromotograms was allowed to proceed 20-22 hours, either in a descending or an ascending direction. Spots were rendered visible by application of a urea solution (5g urea + 20 ml 2N HCl + 100 ml 96% ethanol). Chromatograms were dried at 96 deg for 10 minutes in a drying oven. Under these conditions sucrose and fructose spots become visible [13].

To localize the glucose spot, the paper was sprayed with aniline phthalate (0.93 ml aniline + 1.66g phthalic acid + 100 ml water-saturated butanol). Chromatograms were dried in a drying oven for 5 minutes at 105 deg [14]. As a control, papers were sprayed with a 3% solution of n-anasidine and HCl in n-butanol, which reacts with both reducing and non-reducing sugars to yield colored products. Application of 0.005-ml aliquots of 1% alcohol solutions of sucrose, fructose, and glucose as markers was carried out. After being sprayed, chromatograms were measured by the densitometer fitted with an orange filter for urea and a light blue filter for aniline phthalate.

Use of specially selected light filters made possible differential measurement of sugars on the densitometer.

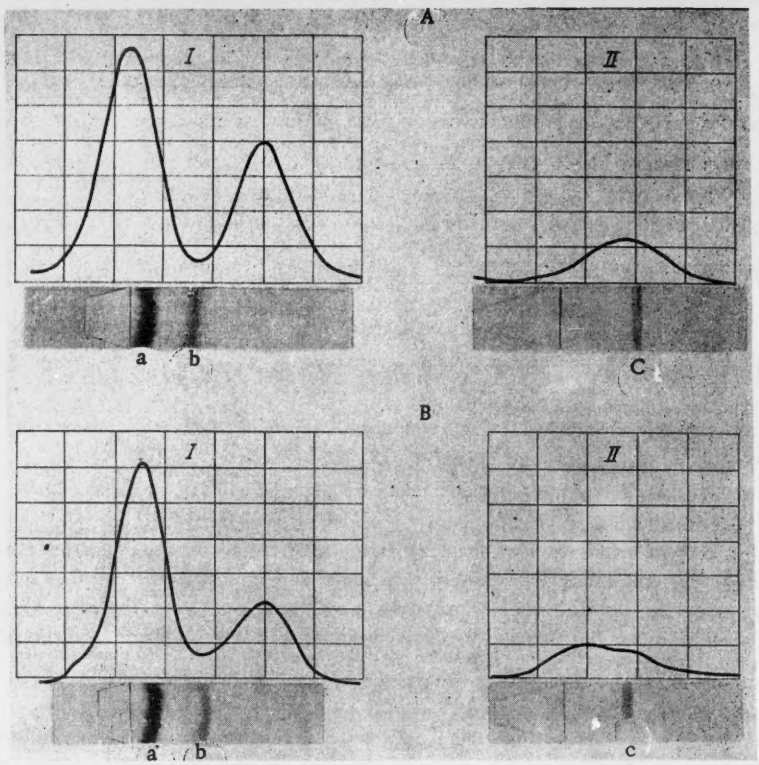

Fig. 1. A,B. (Explanation following page.)

We belive that for qualitative determination of sugars Shipalov's densitometer is highly convenient, since absorption curves vividly show differences in amounts of various individual sugars.

In the course of the study an interesting fact was discovered. If the chromatograms are developed on strips of ordinary shape, tannic substances leave traces and interfere with resolution of sugars. If, however, strips of the shape recommended by Mathias are used, tannic substances rapidly migrate forward and offer no interference to sugar resolution, and sugars can then be easily measured in the densitometer. In our experiments, therefore, we have used only the latter type of strip. Special experiments were performed with samples of fixed green leaves which had not been given a chloroform treatment, but extracted directly with alcohol. It was found that it is fully satisfactory to omit the chloroform treatment.

Determinations were made of sugars in fixed green leaves and in the final product processed by current technilogical methods (control) and by new methods (experimental). In addition, raw samples and processed material from China were also investigated.

RESULTS AND DISCUSSION

Sugars in raw tea leaves and in processed tea from various regions of the USSR. Material analyzed consisted of tea leaves collected in Chakva (Gruz. SSR) and in Matsest (Krasnodar region) and fixed in steam for 5 minutes.

Fig. 1, A and B, shows that in the green leaf sucrose, fructose, and very small amounts of glucose are present, there being more sucrose than fructose. Material from Matsest contains more fructose than that from Chakva. Processed tea samples were obtained in June, 1959, from the Adler Tea Plant. Chromatograms prepared from these samples are shown in Fig. 1, C and D. Tea processed by the usual method (control) contains fructose and glucose, while that processed by the new methods (experimental) contains small amounts of sucrose in addition.

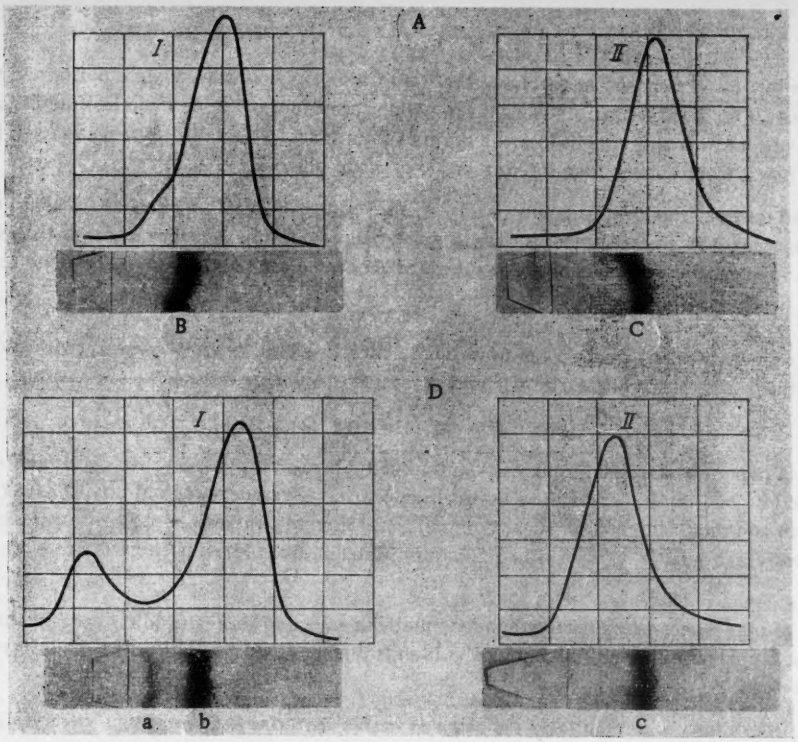

Fig. 1. C, D.

Fig. 1. Chromatograms and absorption curves of sugars from tea leaves and processed tea. A – green leaf from Matsest (Krasnodar region); B – tea leaf from Chakva (Gruz. SSR); C – black tea prepared by usual method; D – black tea prepared by a new method. I – treated with urea; II – treated with aniline phthalate. a – sucrose; b – fructose; c – glucose.

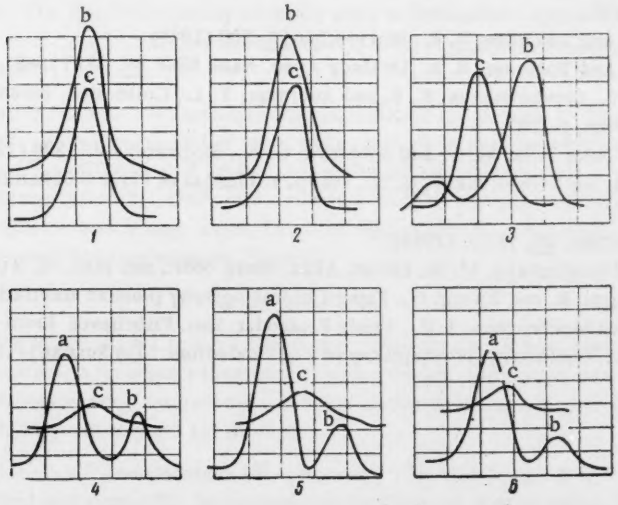

Fig. 2. Absorption curves of sugars in green tea leaves and processed tea from China. 1 – black tea (Kimyn); 2 – black tea (Chuntsin); 3 – black tea (Indo); 4 – aromatic green tea (Chuntsin); 5 – green leaf (Yunan); 6 – green tea (Luntsin). a – sucrose; b – fructose; c – glucose.

Sugars in raw tea leaves and in processed tea from China. These studies were performed with material obtained in 1960. Fig. 2 shows that black tea from various provinces of China contains fructose and glucose, with slight amounts of sucrose being present in some samples from Indo. Green tea contains sucrose, fructose, and glucose, there being more sucrose than fructose and very little glucose.

The green tea leaf contains large amounts of sucrose and very small amounts of fructose and glucose. All three sugars are present in greater amounts than in green tea, indicating that they undergo conversion during processing. Thus, in qualitative sugar composition raw and processed tea from China is the same as that from the USSR.

Of particular interest is processed tea from Indo, which contains in addition to fructose and glucose small amounts of sucrose. This tea is similar to our teas processed in the new manner.

SUMMARY

The use of paper chromatography in conjunction with Shipalov's photoelectric densitometer to investigate sugars in tea yeilds good results. Green tea leaves from Matsest (Krasnodar region) and Chakva (Gruz. SSR) were fixed with steam and then analyzed for sugar composition. Sucrose, fructose, and glucose were found with sucrose occurring in considerably larger amounts than glucose. Material from Matsest contains more fructose than that from Chakva.

Analysis of teas processed by current and new methods revealed that both kinds contain fructose and glucose, while that prepared by the new technology, which is distinguished by high taste and aromatic quality, con tains sucrose as well.

Material from China is substantially the same as native material with respect to carbohydrate composition.

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CONTRIBUTIONS TO THE MECHANISM OF CONCENTRATION AND INCORPORATION INTO PROTEIN OF LYSINE-1-C^{14}, GLYCINE-1-C^{14}, METHIONINE-S^{35}, AND TYROSINE-C^{14} BY ERLICH ASCITES TUMOR CELLS OF MICE

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Amino acid incorporation by cells against a concentration gradient with the expenditure of energy has been studied in detail by Christensen et al. [1-6].

In previous studies [7, 8], we have investigated the relationship between in vitro concentration and incorporation into protein of labeled amino acids by tumor cells and normal cells. In vitro experiments on Erlich ascites tumor cells of mice [8] have shown that at physiological concentrations accumulation of glycine-1-C^{14} and incorporation into protein by tumor cells is proportional to external concentration. At very high external concentrations, internal concentration approaches external concentration. Evidently, active uptake is not involved.

In vitro investigation of the relationship between accumulation and incorporation revealed [8] that for glycine-1-C^{14} and methionine-S^{35} administered to tumor cells there is a parallelism between these two processes, in contrast to normal tissues. The relative capacity of tumor cells to incorporate lysine-1-C^{14} and tyrosine-C^{14} is considerably greater than their relative concentration activity. This suggests that a different mechanism is operative for these amino acids.

In order to elucidate differences in the concentration mechanisms for glycine-1-C^{14}, methionine-S^{35}, lysine-1-C^{14}, and tyrosine-C^{14}, the following studies were made: a study of the competitive interaction of various amino acids with glycine-1-C^{14} and lysine-C^{14} during accumulation and incorporation into protein; a study of the effect of metabolic inhibitors (KCN, 2,4-DNP, NaF, azide); a study of the effect of vitamin B_6 analogues on concentration and incorporation of these amino acids, based on reports that B_6 derivatives, notably pyridoxal, participate actively in amino acid accumulation by the cell.

METHODS

Studies were made using Erlich ascites tumor cells of mice collected 6-10 days after injection. 100 mg of tumor cell material was incubated in Robinson's medium [9] with glucose. Incubation was carried out in Warburg vessels at 37.8 deg for 30 minutes in an O_2 atmosphere. Labeled amino acid was added to the medium on the basis of 10,000-20,000 counts per minute per 2 ml medium.

Concentration of glycine-1-C^{14}, methionine-S^{35}, tyrosine-1-C^{14}, and lysine-1-C^{14} by tumor cells was followed by a method described elsewhere [7]. In the study of the effect of various amino acids on lysine-1-C^{14} concentration, non-radioactive compounds (alanine, cysteine, methionine, glutamine, lysine, glycine, and glutamic acid) were added to the incubation medium to give final concentrations of 10^{-4}, 10^{-3}, and $15 \cdot 10^{-3}$M. Final inhibitor concentrations were as follows: $5 \cdot 10^{-3}$M KCN, $1 \cdot 10^{-3}$ and $2 \cdot 10^{-3}$M DNP, $2 \cdot 10^{-2}$M NaF, and $1 \cdot 10^{-3}$ and $5 \cdot 10^{-3}$M azide.

TABLE 1

The Effect of Some Amino Acids on the In Vitro Accumulation and Incorporation into Protein of Glycine-1-C^{14} by Erlich Ascites Tumor Cells of Mice
Incubation 30 min. in O_2. Values presented obtained in 3-4 experiments

Amino acid added	Final conc. of added amino acid					
	$1 \cdot 10^{-4}$ M		$1 \cdot 10^{-3}$ M		$1{,}5 \cdot 10^{-2}$ M	
	I	II	I	II	I	II
Alanine	82,0	75,2	70,9	78,1	38,7	50,5
Lysine	62,1	80,6	64,5	87,0	83,3	119,0
Cysteine	75,2	89,2	70,9	67,5	63,0	56,4
Methionine	72,4	78,7	54,9	69,4	52,6	58,4
Glutamic acid	77,0	87,7	74,0	100,0	60,0	5,6
Glutamine	70,9	200,0	68,5	135,1	60,0	103,1

Note. In this and following tables: I—amino acid accumulation, % of control; II—protein radioactivity, % of control.

TABLE 2

Effect of Some Amino Acids on the In Vitro Accumulation and Incorporation into Protein of Lysine-1-C^{14} by Erlich Ascites Tumor Cells of Mice
Incubation 30 min. in O_2. Average values from 4-8 expts.

Amino acid added	Final conc. of added amino acid					
	$1 \cdot 10^{-4}$ M		$1 \cdot 10^{-3}$ M		$1{,}5 \cdot 10^{-2}$ M	
	I	II	I	II	I	II
Alanine	90,9	87,2	87,3	82,4	58,4	90,9
Lysine	59,5	82,5	57,3	79,9	28,7	37,5
Cysteine	81,6	89,0	87,5	98,0	67,0	109,3
Methionine	74,5	85,8	58,7	80,8	60,2	87,6
Glutamic acid	92,5	88,9	93,0	96,8	54,2	142,8
Glutamine	51,2	126,5	48,5	127,0	40,7	80,3

TABLE 3

The Effect of Metabolic Inhibitors on the In Vitro Accumulation and Incorporation into Protein of Glycine-1-C^{14}, Lysine-1-C^{14} Tyrosine-C^{14}, and Methionine-S^{35} by Erlich Ascites Tumor Cells of Mice
Incubation 30 min. in O_2 at 37.8 deg.

Amino acid	$5 \cdot 10^{-3}$ M KCN			$5 \cdot 10^{-3}$ M NaCN			$1 \cdot 10^{-3}$ M DNP			$2 \cdot 10^{-2}$ M NaF			Azide								
	No. of expts.	I	II	No. of expts.	I	II	No. of expts.	I	II	No. of expts.	I	II	No. of expts.	$1 \cdot 10^{-3}$ M		No. of expts.	$5 \cdot 10^{-3}$ M				
														I	II		I	II			
Glycine-1-C^{14}	2	38,1	3,7	4	29,4	6,1	6	92,8	4,5	2	93,6	102,7	1	88,6	23,5	6	75,3	30,7			
Methionine-S^{35}	—	—	—	3	35,9	5,0	1	91,9	2,0	—	—	—	—	—	—	—	—	—			
Lysine-1-C^{14}	7	114,0	26,3	—	—	—	7	153,4	6,8	8	99,1	100,2	—	—	—	—	—	—			
Tyrosine-C^{14}	5	67,0	12,3	—	—	—	4	110,8	5,1	—	—	—	2	85,7	47,8	6	83.3	40,5			

Vitamin B_6 analogues—methyl ester of pyridoxine, isonicotine azide, and phthivazidum were added in amounts to give final concentrations of $1 \cdot 10^{-3}$, $5 \cdot 10^{-3}$, and $1 \cdot 10^{-2}$M, phthivazidum being added as a suspension because of its poor solubility in water.

Proteins from the tumor cells were treated in the usual manner after extraction of the free amino acids [10]. Protein radioactivity was measured with an end window counter, using 10 mg dry protein. Results are presented as percentages of values obtained with control samples.

RESULTS

Table 1 contains data on the effect of certain amino acids on concentration and incorporation of glycine--1-C^{14}.

At low concentrations ($0.1 \cdot 10^{-3}$M), alanine, lysine, cysteine, methionine, and glutamic acid retard glycine--1-C^{14} accumulation to about the same extent (20-25%). Incorporation of glycine into protein is retarded in equal degree. At these concentrations, glutamine retards accumulation by 30%, but doubles incorporation.

At 10^{-3}M, the retarding effect on accumulation varies from 29 to 46%, while incoporation is inhibited somewhat less. With glutamic acid, there is no reduction in incorporation, while glutamine increases it somewhat.

Further increase in extracellular concentration of competing amino acids leads to a significant reduction in the cells accumulation capacity. Alanine ($15 \cdot 10^{-3}$M), an amino acid similar to glycine in structure, inhibits its accumulation by 62.3% and reduces incorporation by 50%. The inhibitory action of the other amino acids on glycine accumulation is somewhat weaker, maximum inhibition being 40%. At this concentration, lysine and glutamine are without effect on glycine incorporation, while glutamic acid has a pronounced inhibitory effect; possibly this latter is due to dilution of the label by non-radioactive products of glutamic acid conversion. Consequently, glycine accumulation is inhibited most strongly by alanine at an external concentration many times that of glycine. A parallelism in the effects of competing amino acids on glycine accumulation and incorporation is evident. The only exception is glutamine, which at concentration of $0.1 \cdot 10^{-3}$ and 10^{-3} M inhibits accumulation, but stimulates incorporation.

Investigation of the effect of competing amino acids on accumulation and incorporation of lysine-1-C^{14} (Table 2) revealed that $0.1 \cdot 10^{-3}$M arginine and glutamine reduce accumulation by 41.5 and 48.8% respectively. In contrast to arginine, however, glutamine stimulates incorporation. The other amino acids weakly inhibit both processes.

At a concentration of 10^{-3}M, the effects exerted by the amino acids under consideration are the same as at the lower concentration. At $15 \cdot 10^{-3}$M, all the amino acids except arginine reduce lysine accumulation by 40-60%. All except cysteine and glutamic acid reduce incorporation. Cysteine has no effect on this process, while glutamic acid stimulates it somewhat. Arginine inhibits lysine accumulation most strongly, the amount accumulated being only 28.7% of the control value; incorporation is inhibited to a comparable extent.

With respect to accumulation of both glycine and lysine, therefore, the amino acid which is most strongly inhibitory is similar in structure to the compound whose uptake is inhibited. This type of inhibition may be interpreted as a general criterion of transfer of amino acids with a given steric conformation, and suggest that there are different transfer systems for each class of amino acids.

Glutamine has approximately the same effect on glycine and lysine. At low concentrations it inhibits accumulation but stimulates incorporation. This indicates that the accumulation and incorporation mechanisms are different. Investigation of the effect of metabolic inhibitors on accumulation and incorporation of glycine-1-C^{14}, methionine-S^{35}, lysine-1-C^{14}, and tyrosine-1-C^{14} (Table 3) revealed that KCN and NaCN inhibit glycine accumulation by 62 and 71.6% respectively. At the same time, incorporation was reduced by 96.3 and 93.9%. Methionine-S^{35} accumulation was inhibited by 64.1%. As in the preceding case, however, incorporation was almost completely inhibited. 2,4-DNP inhibits accumulation of glycine and methionine slightly, but inhibits incorporation strongly. Azide ($5 \cdot 10^{-3}$M) inhibits glycine accumulation 24.7% and incorporation 69.3%.

Inhibitors affect accumulation of lysine-1-C^{14} and, in some cases, of tyrosine-C^{14} in different ways. Cyanide has no effect on lysine accumulation, while it reduces tyrosine accumulation 33%. At the same time, incorporation is reduced 4-8-fold. 2,4-DNP enhances accumulation of both compounds, while it inhibits their incorporation. NaF is practically without effect on either process.

TABLE 4

The Effect of Isonicotinazide, Methyl Ester of Pyridoxine, Phthivazidum, and Levomycetin on the In Vitro Accumulation and Incorporation into Protein of Glycine-1-C^{14}, Methionine-S,^{35} Lysine-1-C^{14}, and Tyrosine-C^{14} by Erlich Ascites Tumor Cells of Mice Incubation 30 min. in O_2 at 37.8 deg.

Amino acid	Isonicotinazide						Methyl ester of pyridoxine			P			Levomycetin		
	$5 \cdot 10^{-3}$ M			$1 \cdot 10^{-3}$ M			$5 \cdot 10^{-3}$ M			$5 \cdot 10^{-3}$ M			$5 \cdot 10^{-3}$ M		
	Expts. no.	I	II	Expts. no.	I	II	Expts. no.	I	II	Expts. no.	I	II	Expts. no.	I	II
Glycine-1-C^{14}	4	72,3	88,5	5	77,6	80,2	5	84,4	14,0	5	83,1	101,4	5	46,9	32,0
Methionine-S^{35}	5	102,8	81,1	3	99,4	84,6	4	100,5	61,1	3	106,6	92,9	3	107,1	66,2
Lysine-1-C^{14}	2	106,6	77,1	—	—	—	6	134,6	15,1	6	115,7	100,0	6	77,4	44,9
Tyrosine-C^{14}	4	103,8	71.2	4	96,4	81,5	1	92,3	29,1	1	94,6	79,3	1	100,0	57,5

On the basis of these data, the amino acids may be divided into two groups. In the first, consisting of methionine and glycine, accumulation is strongly inhibited by KCN; 2,4-DNP has a slight inhibitory action, and NaF is substantially without effect. In the second, consisting of lysine and tyrosine, the cyanides either have no effect on accumulation or a very weak inhibitory effect, and DNP stimulates it to some extent. In both groups, KCN and DNP strongly inhibit incorporation.

In the elucidation of the concentration mechanisms characteristic of the two groups, it seemed of interest to study the effect of vitamin B_6 analogues on amino acid accumulation, inasmuch as these have been reported to play a prominent role in amino acid concentration by the cell. Analogues used were isonicotine azide, the methyl ester of pyridoxine, and phthivazidum. The latter was added in the form of a suspension because of low water solubility.

It was found that isonicotine azide inhibits glycine-1-C14 accumulation by 27.7-22.4%. Incorporation into protein is inhibited to almost the same degree (Table 4).

The methyl ester of pyridoxine and phthivazidum inhibit glycine accumulation 15.6 and 16.9% respectively, while the former inhibits incorporation to a marked degree (86%). None of the analogues has any effect on accumulation of lysine, tyrosine, or methionine. The methyl ester of pyridoxine even stimulates lysine accumulation somewhat.

In summary, vitamin B_6 analogues have some inhibitory effect on glycine accumulation, but none on that of lysine, tyrosine, or methionine; this may be an indication that different concentration mechanisms are involved.

DISCUSSION

The competitive interaction of amino acids during their accumulation by the cell has been studied in detail by Christensen et al. [1-6] for ascites tumor cells of mice, by Wiseman [11, 12] for normal tissue cells, and by Cohen [13] for bacterial cells.

It is considered as established that basic amino acids (arginine, lysine) inhibit the accumulation of amino acids of related structure, or of neutral amino acids, but not of acidic amino acids. Thus, arginine reduces glycine accumulation by ascites cells of mice [3], while arginine accumulation can be reduced by another basic amino acid. Accumulation of valine by bacteria is inhibited by leucine or isoleucine, but not by phenylalanine or proline.

In a study of amino acid concentration of the mucous intestine of the hamster [12], it was found that amino acids (methionine, histidine) which are concentrated weakly inhibit accumulation of other amino acids (glycine, proline). At the same time, amino acids (lysine, ornithine) which do not move against a concentration gradient have no effect on accumulation of those for which there is an active transport mechanism.

In view of these interreations between neutral, acidic, and basic amino acids, the hypothesis has been advanced that each class of amino acids has a different transfer system [1]. Our data on the competitive interaction of various amino acids with glycine-1-C^{14} during accumulation of the latter by ascites tumor cells of mice support this hypothesis.

Study of amino acid incorporation into protein by tumor cells shows that it ordinarily parallels accumulation (Table 2). In other words, an effect on incorporation of an amino acid can be attributed to the prior effect on accumulation. On the other hand, glutamine in low concentrations, which reduces accumulation to some extent, stimulates incorporation. Johnston [14] has observed a similar phenomenon. Apparently this is due to direct stimulation of protein synthesis by the amino acid [15].

The effect of metabolic inhibitors on accumulation differs from that on incorporation. The significantly greater cyanide inhibition of glycine incorporation, as compared with its accumulation (96.3% as against 62%), indicates that incorporation is more sensitive to disruption of respiratory processes.

Evidently lysine uptake, and possibly tyrosine uptake, does not require energy, and therefore proceeds by another path. This conclusion is supported by experience with vitamin B_6 analogues as they affect the accumulation of labeled amino acids. The three analogues tested inhibit glycine accumulation 16-25% on the average, but have no effect on lysine and tyrosine accumulation Christensen [4], using another analogue – desoxypridoxine – found that it inhibited glycine accumulation, but not that of phenylalanine. There have been no reports of the effect of this analogue on lysine and tyrosine.

Thus, at present there is no doubt that there are different accumulation mechanisms for various groups of amino acids. Possibly other compounds besides pyridoxal participate in amino acid uptake, in analogy with sugar uptake [16]. The possibility that inorganic ions are involved has not been ruled out.

SUMMARY

Alanine, lysine, cysteine, methionine, and glutamic acid competitively inhibit the accumulation and incorporation into protein of glycine- C^{14} by ascites tumor cells of mice.

Arginine, alanine, cysteine, and glutamic acid inhibit accumulation and incorporation of lysine-C^{14}. Arginine at a concentration of $1.5 \cdot 10^{-2}$M inhibits to the greatest extent (71.3%).

Glutamine depresses accumulation of glycine-1-C^{14} and lysine-C^{14}, but stimulates their incorporation into protein. The latter action is apparently due to a direct effect on protein synthesis.

Cyanide inhibits methionine and glycine accumulation by 64.1% and 62% respectively and reduces their incorporation into protein by 95%. It is without effect on lysine accumulation and has some effect on tyrosine accumulation, while it sharply reduces incorporation of these amino acids into protein.

DNP inhibits glycine and methionine accumulation very little and stimulates lysine and tyrosine accumulation; incorporation of all these compounds is strongly depressed.

Isonicotinazide and the methyl ester of pyridoxine at a concentration of $5 \cdot 10^{-3}$M inhibit glycine accumulation and incorporation by 27.7 and 16% respectively. They have no effect on accumulation of lysine, tyrosine, or methionine, however. It is suggested that there are different mechanisms of uptake for the amino acids studied.

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All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of this issue.

ISOLATION OF QUINIC AND SHIKIMIC ACIDS FROM THE SHOOTS OF THE TEA PLANT

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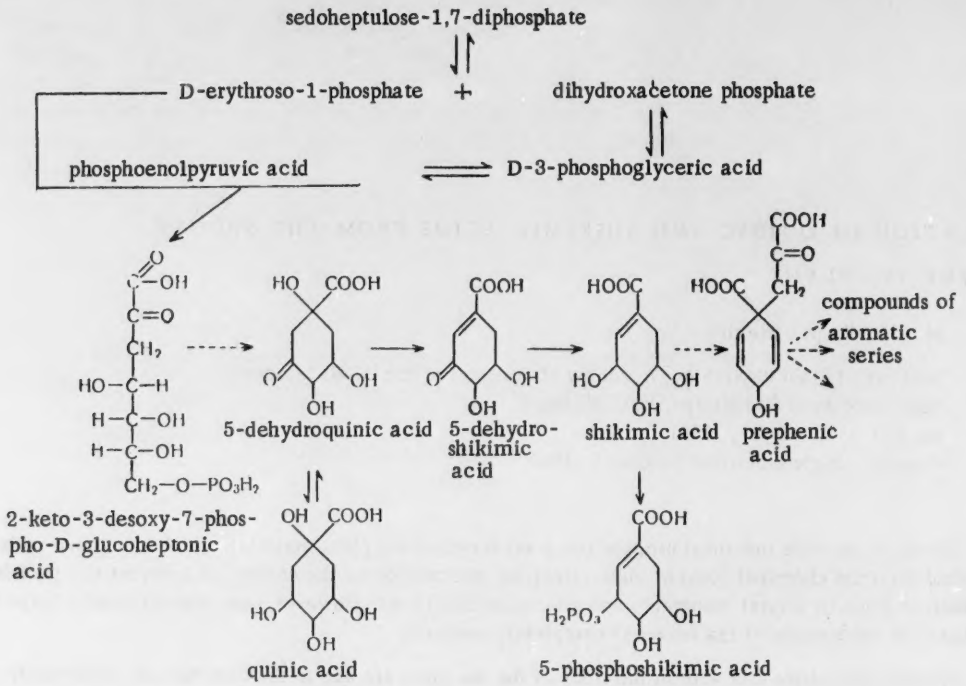
Thanks to its wide industrial use, the tea plant is one of the plant materials which have been most thoroughly studied from the chemical point of view. Detailed information on the content of different compounds in young tea shoots is given in several mongraphs and review articles [1-4]. However, one cannot consider the problem of the chemical constitution of tea leaves as completely resolved.

Special difficulties met with in the study of the tea plant are due to the fact that tea is unusually rich in various phenolic compounds which render the analysis of other components difficult. The following substances have been isolated from tea leaves: seven catechins (DL-catechin, L-epicatechin, L-gallocatechin, L-epigallocatechin, L-epicatechin gallate, L-epigallocatechin gallate, and L-gallocathechin gallate), 15 flavonols (kaempferol, quercetin, myricetin, and their various 3-glycosides), two leucoanthocyanidins (leucocyanidin and leucodelphinidin), and nine simple and composite phenol-carboxylic acids (gallic, m-digallic, ellagic, p-coumaric, caffeic, chlorogenic acids, the isomers of the latter, and p-coumarylquinic and galloyl-quinic acids) [6]. Beside these, tea leaves contain unidentified oxidized (condensed) polyphenols. The phenolic compounds, having different solubilities and being differently precipitated by inorganic salts, considerably complicate the preliminary fractionation of the extracts by usual methods. Apparently, this explains why the presence of considerable amounts of quinic acid, which is demonstrated in the present work, has been overlooked by numerous investigators of tea.

Quantitative data on the amounts of free organic acids (of non-phenolic kind) in tea leaves are not mentioned in the literature. According to Roberts and Wood [7], the freshly prepared juice of tea shoot contains citric and malic acids. Bokuchaeva and Soboleva [8] studied by paper chromatography an etherial extract of dehydrated tea leaves treated with 25% sulfuric acid, and detected oxalic, citric, quinic, malic and fumaric acids.

Quinic acid is specially interesting for the investigator, as it may be an intermediate product in the biosynthesis of the phenolic compounds which are so characteristic for tea. Butkevich [9, 10] has shown long ago that quinic acid is transformed in cultures of Citromyces and Penicillium into polyphenols and in particular, into protocatechuic acid. During a study of the biosynthesis of aromatic amino acids in microorganisms, Davis [11, 12] has proved that one of the basic precursors of the aromatic ring is 5-dehydroquinic acid, which is partly converted to quinic acid. Although quinic acid apparently belongs to a side branch of the amine biosynthetic chain of aromatic compounds in microorganisms, in higher plants it may occupy a central position as well. This is confirmed by its wide distribution in plants in the free state, as well as in an esterified state (chlorogenic, isochlorogenic, p-coumarylquinic, and galloylquinic acids). Infiltration of quinic acid in tea leaves leads to an accumulation of catechins [13]. It has also been shown recently [14] that some higher plants contain an enzymic system which ensures the conversion of quinic acid to shikimic acid (3,4,5-trihydroxycyclohexene-1-carboxylic acid); the latter is already a close precursor of various polyphenols [15-17] with hydroxyl groups in o-position. This acid is formed from sedoheptulose-1,7-diphosphate through a series of intermediate steps [18-20]. Like many other intermediate products, shikimic acid does not usually accumulate in the tissues investigated. Exception are the fruits of the star anise (Illicium religiosum Sieb.) from which Eijkmann [21] was the first to isolate shikimic acid (it contains about



11% of shikimic acid), and the young green pine cones [23] which contain up to 14% of shikimic acid. The presence of small amounts of shikimic acid (of the order of 0.03%) in the leaves of the tea plant has been demonstrated by Zaprometov and Silina [24] by means of paper chromatography carried out with a methanol extract purified by ion-exchangers. As we shall show it below, young tea sprouts contain 1.5% of free quinic acid. If one takes into consideration the fact that tea leaves contain about 1% of galloquinic acid [25,5], and 0.3% of chlorogenic acid [5], and the fact that the leaves also contain isochlorogenic acid and p-coumarylquinic acid, one would be able to conclude that the total quinic acid content (free and esterified) in tea leaves reaches 2.5% of the dry weight. Such a high quinic acid content should attract the attention of those who investiage the biochemical processes occuring during the industrial processing of tea leaves.

The present investigation constitutes a part of our studies on the biosyntnesis of catechins in the tea plant.

EXPERIMENTAL PART*

Trifoliate young shoots ("fleches") of the tea plant, harvested at the end of 1959 on the VNIICh and SK plantation (Anaseuli, Georgian SSSR), were stabilized by vapor with a subsequent drying at 80 deg. The powdered air-dried material (1 kg) while heating on a water-bath was extracted thrice with a mixture of benzene and chloroform (1:1), in order to remove resins, pigments and caffeine from the bulk of the product. The extracted material was then dried by spreading it in a thin layer on filter-paper, and extracted twice with hot 80% methanol. The pooled methanolic extracts were evaporated, diluted with warm water, and filtered. The opalescent filtrate was treated with skin powder (about 700 g). The mixture was carefully stirred until it formed a gruel-like homogeneous mass, and then left to stand overnight.

On the next day, the mixture was filtered with suction on a large Buchner funnel, and the product remaining on the filter was carefully wasned with warm water. The mixed filtrate and washings were treated with 20% $Pb(CH_3COO)_2$ solution (50 ml for each liter of extract; final volume: 7.8 liters). The precipitate which separated out was filtered off and washed with a small amount of water containing a few drops of $Pb(CH_3COO)_2$. The combined filtrate and washings were treated with a concentrated $(NH_4)_2CO_3$ solution for the removal of the greatest part of Pb++ and flavonols. During this operation, the pH of the solution changed from 4.2 to 7.5.

* In collaboration with E. I. Silina

The sediment formed was separated by filtration (volume of the filtrate: 10.5 liters content of dry residue, 172 g). The solution was subsequently acidified with CH_3COOH to pH 5.2. The small amount of precipitate formed was removed and rejected, and the filtrate was passed through a column of the cation-exchanging resin KU-2 (50 x 4.2 cm, in its H^+ -form), and the column was washed with water. Volume of the eluent: 11.7 liters; pH, 2.5; dry residue content, 84.0 g.

Preliminary fractionation of acids on an AN-2f column. The solution obtained as described above was passed through a column of the weakly basic anion-exchanger AN-2f (35 x 3.2 cm, in its CH_3COO^- -form) with a velocity of 80-100 ml/hr, and the column was washed with 2.5 liters of distilled water. The eluate did not give a positive reaction for shikimic acid with the $NaIO_4$-$KMnO_4$ reagent [26]; the test for unvolatile acids was also negative (a few drops of the solution were put on filter-paper, the filter-paper was kept for 5-7 min above a boiling water-bath, dried and sprayed with a solution of bromocresol green).

The organic acids were eluted from the column with 1N NH_4OH solution (elution velocity: 70-80 ml/hr); the eluate being collected in fractions of 50 ml. Soon after elution was started, the eluate became yellow, and then orange and orange-red. At the end of the elution (2 liter of NH_4OH were used), the resin "sintered" appreciably: the height of the column of ion-exchanger diminished from 35 to 25 cm.

The different fractions of the eluate were tested for the organic acid content by the method of ascending chromatography on small strips of paper (20 cm in length). Solvent used: mixture of n-butanol and 85% HCOOH (3:2.5 by volume), development time: 2.5-3.5 hr. The chromatograms were made visible either with $NaIO_4$-$KMnO_4$ reagent [26], or with 0.05% $NaIO_4$ solution followed with the benzidine reagent [27].

Fractions I-IV, and to a smaller extent, fractions VII-X contained an acid which gave the same qualitative test (instantaneous yellow color with $NaIO_4$-$KMnO_4$, and a pink spot appearing after 5 min with benzidine), and the same R_f value as a control of shikimic acid. In fractions I-X, shikimic acid was accompanied by large amounts of another acid, the R_f value and qualitative reactions of which (pale-yellow color with $NaIO_4$-$KMnO_4$ appearing in 7-10 min, and gray-pale blue spot after 5 min with $NaIO_4$ -benzidine) corresponded to those of a control of quinic acid. In all the fractions collected (I-XXIII) there was no organic acids with R_f values higher than that of shikimic acid. From the starting line of the chromatogram up to the location of the shikimic acid spot, the periodate-benzidine reagent revealed lightly colored zones. Starting with fraction XI, this reagent revealed white or yellowish spots only in the neighborhood of the starting line.

Fractions with similar composition were combined as follows: I-VI, containing shikimic acid and quinic acid, and having a dry matter content of 21.7 g; VII-X, containing quinic acid and traces of shikimic acid, with a dry matter content of 9.6 g; XI-XVII, containing acids with very low R_f values, with a dry matter content of 2.5 g; and XVIII-XXIII, containing acids appearing near the starting line on the paper chromatograms with a dry matter content of 2.5 g. The total yield of the fractions I-XXIII was 35.0 g, i.e., 3.5% of the starting material.

It is noteworthy that ion-exchangers, in spite of their being carefully washed, contain a certain amount of extraneous material, which is washed out during the chromatographic runs and appears in the eluate. This is particularly so with the weakly basic anion-exchanging resins, such as AN-2f, and Amberlite IR-45. It has been recently shown [28] that distilled water itself elutes from Amberlite IR-45 more than 20 chemical substances giving a color with ninhydrin reagent. Such contaminating material can be completely removed by a subsequent passage of the eluate through a column with a cation-exchanger containing sulfo-groups (e.g., KU-2, Dowex-50).

Separation of the constituents of fractions I-VI (column No. 1 with the resin Amberlite IRA-400). The combined fractions I-VI were passed through a column of the cation-exchanger KU-2 (30 x 2.1 cm, in its H^+ -form); the column was washed with 150 ml of water. The total dry matter content of the eluate and washings was 20.5 g. The solution thus obtained was passed through a column (36 x 3.2 cm) of the strongly basic anion-exchanger Amberlite IRA-400 (in its CH_3COO^- -form) with a velocity of 120-150 ml/hr. After a thorough washing of the column with water in order to displace all organic acids, gradient elution with acetic acid was applied. For this purpose, the column was connected to a separator fitted with a magnetic stirrer, and containing 0.5 liters of distilled water, which communicated, in turn, with a larger separator containing 2.0 liters of 1.0 N CH_3COOH. The velocity of the elution was 120-150 ml/hr. The eluate, collected in 50 ml fractions, was tested for the presence of substances giving visible reactions with $NaIO_4$-$KMnO_4$ and $NaIO_4$-benzidine reagents. The test were carried out by absorbing two drops of each of the fractions on narrow strips of chromatographic paper, and treating the paper with the mentioned reagents after drying.

A positive reaction with $NaIO_4$-$KMnO_4$ first appeared in fraction 14. Fractions 15-18 gave an instantaneous yellow color with $NaIO_4$-$KMnO_4$, and a pink color with $NaIO_4$-benzidine reagent, characteristic for shikimic acid (after treatment with benzidine: dark fluorescence under ultra-violet light). Fractions 20-74 gave qualitative tests corresponding to those of quinic acid (pale yellow spot with $NaIO_4$-$KMnO_4$, appearing 10 min after application of the reagent; grayish-blue spot with $NaIO_4$-benzidine reagent). Fractions 25-26 gave a positive reaction with $NaIO_4$-$KMnO_4$ (pale yellow color appearing after 15 min); with $NaIO_4$-benzidine, they gave white spots, the central part of which rapidly turned blue.

Fractions 14-19 (total volume: 300 ml) were combined and evaporated in vacuum. The concentrated solution rapidly crystallized. After drying in a vacuum dessicator over P_2O_5 and NaOH, the yield was 3.03 g. Chromatography on paper [solvent: n-butanol–85% HCOOH (3:2.5); reagent: $NaIO_4$-benzidine] revealed the fluorescence of quinic acid, and a considerably smaller amount of shikimic acid.

Fractions 20-24 (volume: 250 ml) were processed in the same manner as fractions 14-19. The product crystallized upon concentration. Yield, 6.21 g. Chromatography on paper revealed the presence of quinic acid and of very small amounts of shikimic acid.

Fractions 25-27 (volume: 150 ml) were also treated in the same manner. Crystallization occured during concentration. Yield, 2.50 g. The chromatogram revealed the presence of quinic acid, and traces of acids with lower R_f values.

Fractions 28-37 (volume: 500 ml) were worked up in the same manner. Crystallization occured during concentration. Yield, 4.58 g. The chromatogram revealed a spot due to quinic acid, merging into that of an unknown substance (yellow color with $NaIO_4$-benzidine reagent) with a slightly higher R_f value, as well as spots due to the presence of acids with low R_f values.

Fractions 38-56 (volume:950 ml) were worked up in the same manner. No crystallization happened upon condensation. After drying in a vacuum dessicator over P_2O_5 and NaOH, the yield was 1.20 g (orange-red resin). The chromatogram revealed the presence of the unknown substance giving a yellow color with $NaIO_4$-benzidine reagent, and of small amounts of acids with low R_f values.

The total yield of fractions 14 to 56 was 17.52 g, i.e., 85.5% of the amount submitted to column chromatography.

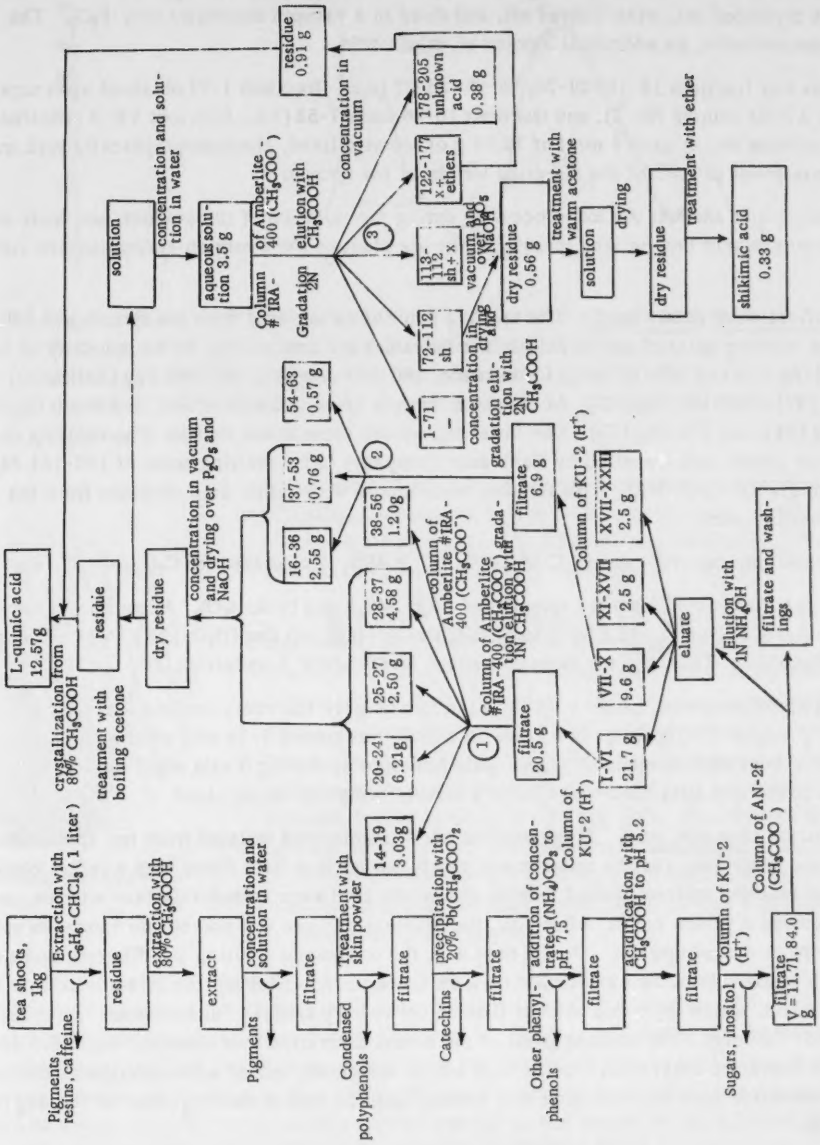
Separation of the constituents of fractions VII-X (column No. 2 with the resin Amberlite-IRA-400). The combined fractions VII-X (eluate from the column with the resin AN-2f) was passed through a column of the cation-exchanger KU-2 (30 x 2.1 cm; in its H^+ -form), and the column was washed with water. The combined eluate and washings contained 6.9 g of dried residue. The solution thus obtained was passed through the column of Amberlite-IRA-400 regenerated after the previous fractionation (36 x 3.2 cm; in its CH_3COO^- -form). Gradient elution technique analogous to that described above was used for the separation of the organic acids. After 1,900 ml of eluent was collected, the column was washed, in succession, with 1.0 liter of 1N HCl, and 0.35 liter of 0.5N HCl (until Cl^- appeared in the eluent). A positive reaction with $NaIO_4$-$KMnO_4$ reagent was first observed with fraction 16.

Fractions 16-36 (volume: 1,050 ml) were worked up as described above. There was no crystallization upon concentration. Yield, 2.55 g. The chromatogram revealed the presence of quinic acid (the spot contains some fluorescing foreign products) and a small amount of shikimic acid, as well as a spot on the starting line, giving a yellow color upon treatment with $NaIO_4$-benzidine reagent.

Fractions 37-53 (volume: 850 ml) were treated in the same manner. No crystallization occured upon concentration. Yield, 0.76 g. The chromatogram revealed the presence of traces of quinic acid, and several weak spots belonging to unknown acids.

Fractions 54-63 (volume: 500 ml), obtained by elution with 0.1N HCl were worked up in the same manner. Yield, 0.57 g. Substances revealed by $NaIO_4$-benzidine reagent were absent on the chromatogram. In the regions corresponding to quinic and shikimic acids, observation under ultraviolet light revealed two dark spots.

The total yield of fractions 16 to 63 was 3.88 g, i.e., 56.3% of the amount put on the column. Further fractions were not studied as they did not give any reaction with $NaIO_4$-benzidine reagent.



For convenience, the successive steps in the separation of the acids are represented in the above scheme:

Isolation of quinic acid from the fractions. After a series of trial, the simplest and easiest method for the isolation of quinic acid from the chromatographic fractions has been elaborated as follows (the fractions contain, together with quinic acid some other acids, and some impurities which probably consist of substances eluted by acetic acid from the anion-exchangers AN-2f and Amberlite-IRA-400):

The dry residues from the different fractions, combined as already described, were treated twice with a little warm acetone. This solvent dissolved almost all the shikimic acid, a considerable amount of impurities, and only a negligible amount of quinic acid.

The residue was then freed from acetone, and dissolved in 80% CH_3COOH; the solution was filtered through a sintered glass filter No. 3, and kept in a refrigerator until it crystallized. In contrast to glacial acetic acid, 80% CH_3COOH was a convenient solvent for quinic acid: the acid dissolved readily and crystallized out rapidly

(when this solvent was used most of the product crystallized out upon standing for 12-18 hr in the cold). The crystals, which separated out, were filtered off, and dried in a vacuum dessicator over P_2O_5. The mother liquors yielded, upon concentration, an additional amount of quinic acid.

In this way fractions 14-19; 20-24; 25-27; 28-37 (i.e., fractions I-VI obtained upon separation on the anion--exchanger AN-2f column No. 1), and fractions 16-36 and 37-53 (i.e., fractions VII-X, obtained upon separation on AN-2f, column No. 2) gave a total of 12.57 g of recrystallized, chromatographically pure quinic acid. This quantity corresponds to 1.3% of the air-dried weight of tea sprouts.

By taking into account the losses occuring during the isolation of the product, one must admit that the content of free quinic acid in trifoliate sprouts of the tea plant of the Georgian variety attains 1.5% of their dry weight.

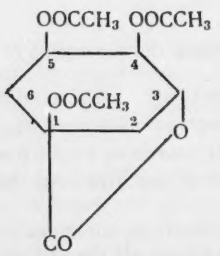
Identification of quinic acid. The melting point of quinic acid from tea sprouts was 180.5-181.5 deg. Data given for the melting point of quinic acid in the literature are conflicting: in the majority of cases the values given are of the order of 160-167 deg, for instance, 160-161 deg [29], 161-162 deg (Beilstein), 162 deg [30, 31], 165-66 deg [32], 166-168 deg [33]. At the same time, a few authors have obtained much higher values, such as 183-184 deg [34], and 184 deg [35]. The latter figures are close to our values. The melting point of a commercial sample of quinic acid (supplied by Kahlbaum Company) had a melting point of 180-181 deg after recrystallization from glacial CH_3COOH. This sample, when mixed with quinic acid obtained from tea sprouts, did not depress its melting point.

Elemental microanalysis gave: C 44.06%, H 6.48%. Calculated for $C_7H_{12}O_6$: C 43.71%, H 6.29%.

Quinic acid extracted from tea sprouts had $[\alpha]_D^{25}$ -42.8 deg (c 6, H_2O). According to the literature, $[\alpha]_D^{15}$ -43.8 deg (c 6, H_2O); $[\alpha]_D^{25.5}$ -42.1 (c 5, H_2O) (Beilstein); $[\alpha]_D^{23}$ -43 deg (H_2O) [30]; $[\alpha]_D$ -43 deg (H_2O) [35]; $[\alpha]_D^{20}$ -42 ± 2 deg (H_2O) [31]. Thus, the tea plant contained levorotatory L-quinic acid.

On the chromatograms, quinic acid from tea sprouts gave the characteristic reactions for quinic acid: with $NaIO_4$-$KMnO_4$ reagent [26] (pale yellow color on a pink background 7-10 min after application of the reagent), and with $NaIO_4$-benzidine reagent [27] (gray-pale blue spot appearing 5 min after application of the reagent). The position of the spot coincided with that of a control sample of quinic acid.

Acetylation of quinic acid. Eight hundred mg of quinic acid isolated from tea sprouts were treated with 10 ml of acetic anhydride, and the mixture was gently boiled in a flask fitted with a reflux condenser for 3 hr. Soon after the reaction mixture started to boil, the quinic acid went completely into solution, and the solution gradually acquired a yellow color. After 3 hr, the reaction mixture was poured into 5 times its volume of cold water, and kept in the refrigerator. On the next day, the opalescent solution was filtered, and the filtrate concentrated to a small volume by evaporation on a water-bath. After letting the mixture to stand in the cold, the crystalline product, which separated out was filtered off and crystallized, in succession, from boiling ethanol and acetone (yield: 320 mg). The melting point of the acetyl derivative thus obtained was 215.5 deg (sublimes). According to the literature data, upon heating with acetic anhydride, quinic acid undergoes simultaneously acetylation and lactonization with the formation of a triacetylquinide with a melting point of 131 deg [29], or 132-134 deg (Beilstein):



The acetyl derivative obtained under analogous conditions from the commercial sample of quinic acid melted at 215 deg and gave no melting point depression upon mixing with the acetyl derivative of quinic acid from tea sprouts. The reason of the discrepancy between our data and those given in the literature was not under-

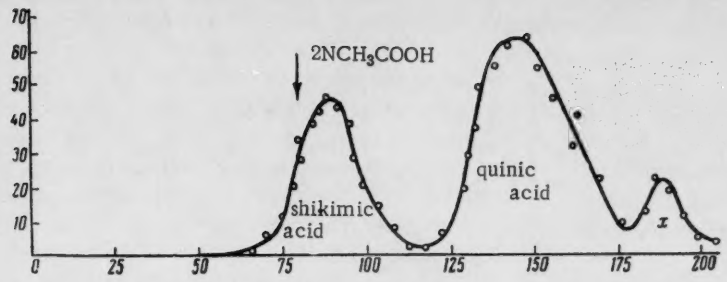


Fig. 1. Elution curve for the column of strongly basic anionic exchanger Amberlite-IRA-400 (in its CH_3COO^- -form; column No. 3)
Axis of abscissas—fraction number (each fraction: 12.5 ml).
Axis of ordinate—intensity of color produced with the periodate-benzidine reagent (in arbitrary units).

Fig. 2. Benzylamine shikimate, X 56.

stood. After at 10-15 min boiling with acetic anhydride in the presence of $ZnCl_2$, L-quinic acid gave tetraacetyl-L-quinic acid, which also had a lower melting point, viz.. 132-136 deg (Beilstein). The acetyl derivative obtained by us has $[\alpha]_D^{25}$ -44.7 deg (c 1.3, H_2O).

Preparation of quinolactone. L-quinic acid from tea sprouts (1g) was heated on a metal bath at 220 deg for 30 min. The product of the reaction was then dissolved in hot ethanol, the solution clarified with activated charcoal, filtered and evaporated to a small volume. Two recrystallizations from ethanol gave colorless crystals with poorly characterized facets, melting at 170 deg. According to the literature, quinolactone melts at 198 deg [36], or at 172 deg, with subsequent solidification and further melting at 187 deg [37]. The melting point of quinolactone obtained from the commercial sample of quinic acid was 171 deg; there was no depression of melting point upon mixing the latter with the quinolactone obtained from quinic acid isolated from tea sprouts. Quinolactone is optically inactive.

Isolation of shikimic acid (column No. 3 with the resin Amberlite-IRA-400. Acetone extracts of fractions 14-19; 20-24 (obtained during the chromatographic separation on a column with Amberlite-IRA-400 of the fractions I-VI, which were eluted from an AN-2f column), and fractions 16-36 (obtained during the separation on Amberlite-IRA-400 of the fractions VII-X, which were eluted from an AN-2f column), as well as the mother liquids of the same fractions, resulting after removal of quinic acid were evaporated in vacuum, and the residue was dissolved in 200 ml of water. The dry weight of this residue was 3.5 g, and the pH of the solution, about 2.3. The solution did not contain any substance absorbable on the cation-exchanger KU-2.

The solution thus obtained was passed through a regenerated column of Amberlite-IRA-400 (in its CH_3COO^- -form ; 43 x 3.2 cm) at a velocity of 120-150 ml/hr. The column was then washed with 750 ml of distilled water, and gradient elution with acetic acid was started.

The column was connected with a separator fitted with a magnetic stirrer and containing 0.5 liters of water, which communicated , in tubes, with a larger separator containing 1.5 liter of 1N CH_3COOH. After this acid was spent, it was replaced by 1 liter of 2N CH_3COOH. The eluate was collected in fractions with the help of a mechanical collector in 12.5 ml test-tubes. Number of fractions: 205; total volume of the eluate: 2.6 liters. The separation of the acids attained by this procedure is represented in Fig. 1. The first acid to be eluted was shikimic acid. After a short but sharp interval, it was followed by quinic acid. The latter was followed almost without transition by an unidentified acid giving an orange-yellow color with $NaIO_4$-benzidine reagent. Fraction collection was started only when the eluate began to give a weak uncharacterized reaction with the $NaIO_4$-$KMnO_4$ reagent (a pale yellow ring around the location of the spot on filter paper, appearing 10-15 min after application of the reagent). The preceding, so as to speak "empty" fractions were rejected.

Compostion of the fractions. Fractions 1-71 (900 ml), upon chromatographic investigation, did not show the presence of substances giving characteristic reactions with $NaIO_4$-$KMnO_4$ and $NaIO_4$-benzidine reagents. They were not given further consideration. Fractions 72-112 (515 ml) were combined and evaporated in vacuum. After drying in a vacuum dessicator over P_2O_5 and granulated NaOH, the yield was 0.56 g. The product gave a positive reaction with $NaIO_4$-$KMnO_4$ and $NaIO_4$-benzidine for shikimic acid. Chromatography on paper (solvent: n-butanol–85% HCOOH (3:2.5); reagent $NaIO_4$-benzidine) revealed one spot, which corresponded to shikimic acid. Fractions 113-121 were rejected; paper chromatography showed that these fractions contained small amounts of shikimic and quinic acids. Fractions 122-177 (704 ml) were combined and evaporated in vacuum. After drying in a vacuum dessicator over P_2O_5 and NaOH, the weight of the residue was 0.91 g. This product gave a positive reaction for quinic acid when tested with $NaIO_4$-$KMnO_4$ and $NaIO_4$-benzidine reagents. Paper chromatography gave a basic spot of quinic acid contaminated with an impurity, which was the cause of a pale-blue fluorescence in ultraviolet light after treatment with $NaIO_4$-benzidine. Beside this, the chromatogram revealed the presence of small amount of substances with high Rf values giving a badly characterized dark coloration with $NaIO_4$-benzidine. Fractions 178-205 (310 ml) were combined and evaporated in vacuum. After drying in a vacuum dessicator over P_2O_5 and NaOH, the product weighed 0.38 g. The spot test of this substance on paper with $NaIO_4$-benzidine reagent gave an orange-yellow color (red-orange fluorescence under ultraviolet light). Paper chromatography revealed the presence of a spot with a Rf value of little below that of quinic acid. This substance gave an orange-yellow color with $NaIO_4$-benzidine.

Attempts to crystallize this substance from 80% CH_3COOH or ethanol were deceptive. The substance was not studied further. The total yield of the fractions obtained was 1.85 g, i.e., 53% of the weight of the product put on the column.

Separation of quinic acid from fractions 122-177. The dry residue (0.91 g) was dissolved in hot 80% CH_3-COOH. After filration through a sintered glass filter No. 3, the solution was kept in a refrigerator. After a long time (25 days), 220 mg of quinic acid crystallized out.

Isolation of shikimic acid from fractions 72-112. The product from these fractions (0.56 g) was dissolved with heating, in glacial acetic acid. The solution thus obtained was filtered through a sintered glass filter No. 3 and kept in a refrigerator. No crystallization was observed even after standing for 12 days in the cold. Therefore, acetic acid was removed in a vacuum dessicator over NaOH, and the residue was repeatedly treated with warm acetone. Paper chromotographic test showed that practically all the shikimic acid was present in the acetone extract. The combined acetone extracts were concentrated in a current of warm air, and the residue was washed twice with small portions of ether for the removal of impurities. During this treatment the shikimic acid was converted from a glassy mass to a white powder. Yield: 0.33 g (0.033% of the air-dried weight of tea sprouts). By taking into account the losses occuring during the isolation procedures, one may say that the content of shikimic acid in the trifoliate sprouts of the tea plant of the Georgian variety attains 0.035% of the dry weight.

Identification of shikimic acid. The melting point of the shikimic acid isolated as described above was 182.5-183.5 deg. According to the literature data, shikimic acid melts at 183 deg [38]; 182.5 deg [39]; 183.5--184.5 deg [40]. The acid obtained from tea sprouts did not show any depression of melting point when mixed with an authentic specimen of shikimic acid (supplied by L. Light and Co., Ltd.) This substance gave the following qualitative tests characteristic for shikimic acid: 1) with $NaIO_4$-$KMnO_4$ (instantaneous yellow color) [26], 2) with $NaIO_4$-benzidine (white spot on a pale blue background, turning pink in 5-10 min, 3) with $Na_2B_4O_7$–KI-H_3BO_3–soluble starch reagent (white spot on a gray-blue background) [41], 4) with sodium nitroprusside–piperazine (bright yellow spot after heating at 100 deg for 5 min) [42].

Analysis values: C 48.52%. Calculated for $C_7H_{10}O_5$: C 48.23%, H 5.80%.

Upon chromatography both in acidic and basic solvents, the substance isolated from tea sprouts gave spots coinciding with those given by the authentic speciment of shikimic acid. Rf values: 0.32 (n-butanol–85% HCOOH–water, 95:10:20); 0.19 (isoamyl alcohol–85% HCOOH–water, 100:23:77); 0.22 (propanol–conc. NH_4OH, 70:30); and 0.41 (ethanol–conc. NH_4OH–water, 80:10:10). The ultraviolet spectrum of the acid obtained from tea sprouts fully coincided with that of the authentic sample of shikimic acid [24].

Preparation of benzylamine shikimate. Shikimic acid isolated from tea leaves (330 mg) was dissolved in hot ethanol, and the solution thus obtained was treated, while stirring, with an alcoholic solution of benzylamine (250 mg). As the product did not crystallize out upon standing in the cold for 24 hr, the solution was evaporated

in vacuum to a syrupy consistency. During evaporation a fluffy precipitate began to separate out. Under the microscope, it appeared as spherical formations, inside which, in some cases, one could distinguish needle crystals. After two recrystallizations from alcohol, the product gave the pure benzylamine shikimate acid as colorless large needle-shaped clusters (Fig. 2) melting at 133-133.5 deg (when rapidly heated to 120 deg); yield, 260 mg. The melting point of benzylamine shikimate prepared in the same way from an authentic specimen of shikimic acid was 134 deg (when rapidly heated to 120 deg). There was no depression of melting point upon mixing the benzylamine shikimates prepared from the tea leaf shikimic acid and the commercial sample.

Regeneration of free shikimic acid from its benzylamine salt by means of the ion-exchange column. Two hundred mg of the benzylamine salt of shikimic acid from tea sprouts were dissolved in 15 ml of warm water, and the solution thus obtained was passed through a small column of the cation-exchange resin KU-2 (12 x 1.2 cm, in its H^+ -form). The column washed with 20 ml of water, and the acid eluate and washings were combined and evaporated in vacuum. Yield, 96 mg. The substance thus obtained was a chromatographically uniform specimen of shikimic acid.

SUMMARY

The methanol extract of young trifoliate shoots of the tea plant of the Georgian variety was treated with skin powder and $Pb(CH_3COO)_2$ to remove polyphenols. In the solution thus obtained 3% organic acids of non-phenolic nature have been found by ion exchange chromatography (columns of KU-2 and AN-2f resins). From the mixture of acids L-quinic acid, m.p. 180.5-181.5°, $[\alpha]_D^{25}$ −42.8° (c6, H_2O) has been obtained by chromatography on a column of the strongly basic anion-exchange Amberlite IRA-400 resin (the CH_3COO^- -form). An acetyl derivative of quinic acid, m.p. 215.5° (with subsequent sublimation) was obtained ($[\alpha]_D^{25}$ - 44.7° (c 1.3, H_2O)), and in optically inactive quinolactone, m.p. 170°. The chromatographic behavior and the qualitative reactions of the isolated quinic acid coincide with the respective properties of a standard sample of L-quinic acid.

The content of free quinic acid in the tea shoots amounts to 1.5% on a dry weight basis. The total content of free and ester bound quinic acid in the tea shoots amount to 2.5% on a dry weight basis.

By a similar procedure shikimic acid has been isolated from the shoots, m.p. 182.5−183.5°. Benzylamine shikimate has been obtained, m.p. 133−133.5°. The UV-spectrum, the chromatographic constants for some solvents, and the qualitative reactions of the isolated shikimic acid coincide with the respective characteristics of a standard sample of shikimic acid.

The content of shikimic acid in the shoots amounts to 0.035% on a dry weight basis.

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ADENOSINE TRIPHOSPHATASE IN VARIOUS CELLULAR FRACTIONS OF THE CEREBRUM

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The presence of adenosine triphosphatase in the brain was first established by DuBois and Potter in 1943 [1]; from their results, and also from the results of Potter and Liebl [2], the ATP-ase activity is lower in brain than in other tissues; DuBois and Potter found that brain ATP-ase, like ATP-ase of other tissues, is activated by Ca^{++}.

Meyerhof and Wilson [3] found that brain ATP-ase is activated by Mg^{++}. The activating effect of Ca^{++} and Mg^{++} on brain ATP-ase was studied by Palladin and Shtutman [4], Épel'baum and co-workers [5], Binkley and Olson [6], and also by Gore [7], and conflicting results were obtained.

In all these investigations, as also in the investigation of Zbarskii and Brisher [8], ATP-ase activity was studied in homogenates or extracts of brain tissue. The first attempt to clarify the distribution of ATP-ase among the cellular structures of brain was undertaken by Abood and co-workers [9]. By differential centrifugation they separated the grey matter of the cerebral hemispheres and the white matter of the spinal cord into nuclei, mitochondria, and supernatant, and found that the highest ATP-ase activity was in the mitochondrial fraction. Cseh and co-workers [10] also found that the mitochondrial fraction had the highest ATP-ase activity. Jordan and March [11] showed that ATP-ase activity in intracellular fractions of rat brain changes with growth.

Thus, published results about brain ATP-ase are few and often contradictory; the evidence about the distribution of ATP-ase among various intracellular elements of cerebral tissue is quite unsatisfactory; and there have been no investigations of the properties and features of ATP-ase from the various cellular fractions of the cerebrum. The methods of differential centrifugation used by the above-mentioned authors to separate different cellular structures were variable and not worked out sufficiently, so that comparison of their results is very difficult. This situation impelled us to attempt a more detailed study of the distribution of ATP-ase among the separate cellular fractions of cerebral tissue, its activity in these fractions and some of its properties, and principally, its activation by calcium and magnesium ions.

METHODS

Differential centrifugation. The studies were carried out on the cerebrum (cerebral hemispheres and cerebellum) of adult rabbits. Separate cellular fractions of cerebral tissue were obtained by differential centrifugation of brain homogenates. The basis of the fractionation procedure was the method suggested by Brody and Bain [12], with the following modifications.

The brain was extracted from the cranium soon after decapitation of the animal, freed of vascular membranes in the cold, and rapidly cut up with scissors into a more or less homogeneous mass. 5 g of minced tissue was homogenized with nine volumes of 0.25M sucrose solution containing 0.0018 M $CaCl_2$ in a manual homogenizer, constructed along the lines of the model of Downs but with a plastic plunger. 40 ml of the resulting homogenate were used for fractionation by differential centrifugation. All stages of fractionation were carried out in the cold by using refrigerated high-speed centrifuges.

TABLE 1

Distribution of Protein among Cellular Fractions of Cerebral Tissue of Rabbits.
As percentages of the total protein content of the original tissue homogenate

	Nuclei	Mitochondria	Microsomes	Soluble fraction	Rinse fluid	Total
	8,6	24,6	1,5	20,0	42,3	97,0
	6,3	21,4	1,4	22,7	45,4	97,2
	6,1	23,3	1,5	22,5	45,1	98,5
	8,6	22,8	2,1	21,9	41,8	97,2
	7,7	24,9	2,1	23,2	40,6	98,5
	8,7	23,8	2,4	21,9	38,2	95,0
	6,8	22,1	2,6	22,1	43,8	97,4
Average	7,5	23,3	1,9	22,0	42,5	97,2

TABLE 2

Adenosine Triphosphatase Activity in Cellular Fractions of Cerebral Tissue (Sucrose Used during Homogenizing Contained $CaCl_2$). As µg P per mg protein after 15 minutes at 37 deg

	Homogenate	Nuclei	Mitochondria	Microsomes	Soluble fraction	Rinse fluid
	22	17	36	57	0	11
	17	20	20	28	1	8
	20	16	22	35	3	8
	21	15	33	63	5	11
	19	25	34	59	3	11
	18	24	29	40	0	14
Average	19	19	29	47	2	11

TABLE 3

Adenosine Triphosphatase Activity in Cellular Fractions of Cerebrum in the Absence of Activators and during Activation by Magnesium and Calcium Ions.
As µg P per mg protein after 15 minutes at 37 deg.

	Homogenate			Nuclei			Mitochondria			Microsomes			Soluble fraction			Rinse fluid		
	Without activators	0,005 M Mg^{++}	0,005 M Ca^{++}	without activators	Mg^{++}	Ca^{++}	without activators	Mg^{++}	Ca^{++}	without activators	Mg^{++}	Ca^{++}	without activators	Mg^{++}	Ca^{++}	without activators	Mg^{++}	Ca^{++}
	18	59	21	9	48	28	23	77	27	16	90	37	3	14	3	8	40	9
	19	59	23	10	46	26	31	84	33	16	95	51	3	11	3	11	41	11
	16	49	19	7	36	21	22	66	24	15	86	42	4	9	4	7	29	6
	22	62	22	15	54	38	32	101	34	16	88	35	—	6	5	16	51	19
	16	57	16	23	58	44	21	79	25	15	60	24	8	12	8	16	39	12
	12	36	17	10	39	28	18	52	23	—	—	—	3	8	3	14	40	13
	15	43	17	16	43	24	21	60	22	16	64	30	3	6	3	12	34	12
	12	36	13	14	37	23	19	42	19	14	62	27	3	5	2	10	29	8
Average	16	50	19	13	45	29	23	70	26	15	78	35	4	9	4	12	38	11

The first centrifugation was carried out at 900 g for ten minutes. The result was a large, very loose sediment containing nuclei, fragments of nerve fibers, and, to a certain extent, mitochondria. The supernatant (initial centrifugate), containing mitochondria, microsomes, and the soluble cytoplasmic fraction, was carefully poured off, and the sediment was suspended in 8 ml of 0.25 M sucrose solution containing 0.0018M $CaCl_2$, and again centrifuged at 900 g for ten minutes. The resulting sediment was rinsed again in the same manner. The initial centrifugate and both rinse fluids were combined and used further for separation of the mitochondria and microsomes.

Nuclei were separated from the sediment by suspending it in 1 M sucrose solution containing 0.0018 M $CaCl_2$ and centrifuging at 2500-3000 g. To obtain a relatively pure nuclear preparation, the same rinse, i.e., repeated resuspension and centrifugation, was carried out 7-9 times. In this way the nuclear fraction was obtained.

All the rinse fluids, containing fragments of nerve fibers, some large mitochondria, and also a small number of nuclei were pooled and subjected to further investigation along with other fractions; in the tables they are designated "rinse fluid".

Mitochondria were sedimented by centrifugation at 18,000-20,000 g for 20 minutes. The sediment was rinsed in 0.25 M sucrose solution and repeatedly centrifuged, thus yielding the mitochondrial fraction.

Both supernatants after sedimentation and rinsing of the mitochondria were pooled, and the microsome fraction separated from them by centrifugation at 44,000-48,000 g for 60 minutes. The result was four cellular frac-

tions (nuclei, mitochondria, microsomes, and soluble fraction) and the above-mentioned fluid rinsed free of nuclei. ATP-ase activity was determined in all fractions.

Estimation of ATP-ase activity. Estimation of ATP-ase activity was carried out in the following manner. The reaction mixture consisted of 2.1 ml of 0.05 M veronal buffer at pH 7.4; 0.1 ml of activator (0.18 M $CaCl_2$ or $MgCl_2$) or water if the experiment was conducted without activator; and 0.4 ml of 0.009 M solution of the sodium salt of ATP. After this mixture had been kept in a thermostat at 37 deg for 15-20 minutes, 1 ml of a suspension of one or another fraction was added, equivalent to 0.5-0.9 mg of protein for the nuclear fraction and 1.0--1.7 mg of protein for the homogenate and mitochondrial, microsomal, and soluble fractions. The final volume of the reaction mixture was thus 3.6 ml. The reaction mixture was incubated for 15 minutes at 37 deg. At the end of this period the reaction was stopped by addition of 0.9 ml of 20μ TCA. The phosphorus split off of ATP by ATP-ase was estimated by the method of Fiske and Subbarow. ATP-ase activity was expressed as μg inorganic phospate, split off to ATP after 15 minutes at 37 deg per 1 mg of protein.

Estimation of protein content. Since the most suitable method to compute enzymatic activity in nerve tissue is to compute enzymatic activity per unit of protein, we first of all determined the protein content in the separate cellular fractions. After trying various methods for estimating protein, we finally settled on the biuret reaction with the modification of Gachev [13], further modified as follows.

Protein of cellular fractions was precipitated by TCA and freed of lipids by extraction first with a cold mixture of alcohol and ether in the ratio of 1:3, then in the ratio of 3:1 (each extraction lasted 60 minutes). After the extraction the proteins were dissolved in 3% NaOH, and the solution was warmed for 30 minutes in a water bath at 60 deg. Then the solution was cooled and its volume was made up to 5 ml with 3% NaOH and 0.15 ml of 20% $CuSO_4$. After 30 minutes, the solution was centrifuged and subjected to photometry. Proteins of blood serum were used as the standard. In order to study the distribution of protein among the various cellular fractions of cerebral tissue, all stages of differential centrifugation were carried out with quantitative retention of the fractions. This allowed one to compute the protein content in various fractions as percentages of the total content in the tissue (in the tissue homogenate).

RESULTS AND DISCUSSION

The results of estimations of protein content are set out in Table 1. Of the four cellular fractions (Table 1), the mitochondria take first place as regards protein content and contain more than 20% of the protein. There is almost as much protein in the soluble fraction. Protein is significantly lower in the nuclear fraction (by about three times), and there is still less protein (be about ten times) in the microsomes. A high protein content was observed for the rinse fluid. Since the rinse fluid contains, in addition to fragments of nerve fibers, a certain amount of large mitochondria, one can confidently say that the mitochondrial fraction contains most of the total protein.

Our results concerning the distribution of protein among the cellular fractions are not in agreement with results in the literature and notably with the results by Clouet and Richter [14]. According to their results, most of the total protein belongs to the nuclear fraction of rat brain (over 50%). However, one must keep in mind that what they termed the nuclear fraction was the sediment left after on centrifugation at 5000 g for 15 minutes. It cannot be doubted that this fraction contains many fragments of nerve fibers and mitochondria; hence one may not consider this fraction as pure nuclei. Clouet and Richter themselves pointed out that this fraction corresponded approximately to nuclei and cell fragments. In consequence, we considered it essential to purify the nuclei as far as possible from secondary contaminants by repeated rinsing.

Since some loss occurred to the nuclear sediment during repeated rinsing, our results for the protein content in the nuclear fraction may be considered somewhat low, but they are doubtless closer to the true values than results obtained without rinsing the nuclei. The significant fraction of the cellular protein contained in the rinse fluid cannot be assigned to nuclear protein; this is principally protein of fragments of nerve fibers and large mitochondria. Hence one must assume that the mitochondria, as compared to the nuclear, microsomal, and soluble fractions, stand first in protein content. Clouet and Richter found more protein in the microsomal fraction than we did (6% vs. 2%); but they sedimented the mitochondria at 12,000 g, rather than 18,000 g, as we did. If one compares the distribution of protein among the four principal fractions and disregards the protein of the rinse fluid, then the mitochondria have 43% of the protein, the soluble fraction 40%, the nuclei 14%, and the microsome 3%.

Studies of ATP-ase activity in separate fractions showed that ATP-ase occurs in all fractions. The microsomal fraction had the highest ATP-ase activity (Table 2). The second highest ATP-ase activity after the microsomal fraction was in the mitochondrial fraction, then the nuclear fraction. The soluble fraction showed very little ATP-ase activity. Since the nuclear fraction is not completely pure nuclei, this might raise doubts as to whether the ATP-ase activity in the nuclear fraction is increased by the impurities in this fraction. However, since the activity of the rinse fraction, which acquired the impurities that might be in the nuclear fraction, was significantly lower, one must conclude that the ATP-ase activity found in the nuclear fraction is really a property of nuclei.

Thus, we found in these experiments that the highest ATP-ase activity is in the microsomal fraction, while, according to the studies of Abood and co-workers [9] and also Cseh and co-workers [10], the highest ATP-ase activity is in the mitochondrial fraction. It became necessary to explain the reason for the disagreement between these results.

In our experiments the sucrose solutions used for homogenizing brain tissue and for differential centrifugation contained $CaCl_2$. The solutions used by the above-mentioned authors did not contain $CaCl_2$. Hence one might suppose that the difference in the results of our experiments was due to the activating effect of Ca^{++} on ATP-ase in our experiments, and this activation might not be the same in the various cellular fractions.

To test this assumption, we conducted experiments to test the activity of ATP-ase in the absence of Ca^{++}, and also experiments to study the effect of Ca^{++} and Mg^{++} on ATP-ase activity. During these experiments it was necessary to exclude $CaCl_2$ from the sucrose solutions used during homogenizing and fractionation, although this had harmful effects on the condition of the nuclei. Since absence of $CaCl_2$ has an effect upon the sedimentation pattern during centrifugation, we changed conditions of centrifugation somewhat. In these experiments the first centrifugation of the homogenate was carried out at 1300 g for 10 minutes. The sediment was rinsed with 0.25 M sucrose solution, and the mitochondria were sedimented from the pooled supernatants by centrifugation at 18,000--20,000 g for 20 minutes. Since the resulting sediment was very loose and difficult to separate from the supernatant, it was subjected to repeated centrifugation at 26,000 g.

The microsomes were separated, as before, by centrifugation at 44,000-48,000 g for 60 minutes. The nuclei were purified by being resuspended 3-4 times in 0.7 M sucrose solution with repeated centrifugation at 2000 g for 20 minutes.

The results of experiments designed to study ATP-ase activity in various cellular fractions in the absence of $CaCl_2$ are set out it Table 3. Here we obtained results similar to the results of Abood and Cseh: we found the highest ATP-ase activity in the mitochondrial fraction; the least activity was detected in the fractions of the microsomes and nuceli, in which the activity was almost identical. Very low ATP-ase activity was again observed in the soluble fraction in these experiments.

Results of experiments designed to study activation of cerebral ATP-ase by Ca^{++} and Mg^{++} are set out in Table 3. Calcium has unequal effect on the ATP-ase of different cellular structures. It has almost no effect on mitochondrial ATP-ase but activates microsomal ATP-ase significantly, by a factor of more than two. This may explain the high ATP-ase activity we observed in microsomes in the first series of experiments, when the sucrose solutions contained $CaCl_2$. Ca^{++} also activates nuclear ATP-ase, but has no effect on the ATP-ase of the soluble fraction. These results of experiments designed to study the activating effect of Ca^{++} compel one to believe that the different cellular fractions contain ATP-ases with dissimilar properties.

As for Mg^{++}, it has an activating effect on ATP-ase of all three cellular structures—nuclei, mitochondria, and microsomes—but to different extents, as is readily apparent from the results in Table 3. Magnesium has the largest activating effect on microsomal ATP-ase (by about five times); Mg^{++} increases ATP-ase activity in mitochondria and nuclei by, on the average, a factor of three. Thus, even the experiments with activation of ATP-ase by magnesium compel one to believe that the various cellular fractions contain ATP-ase of dissimilar properties.

The dissimilar response to Ca^{++} and Mg^{++} on the part of ATP-ases, contained in different cellular structures and the soluble fraction of cerebral tissue, may also provide the reason why some authors found an activating effect of Ca^{++}, and other, an activating effect of Mg^{++} and no effect of Ca^{++}, during studies of ATP-ase activity in homogenates and extracts from cerebrum.

The distribution of ATP-ase activity among cellular fractions of liver and the influence of Ca^{++} and Mg^{++} on liver ATP-ase were studied in rats by Novikoff and co-workers [15]. They found that mitochondria have the highest ATP-ase activity, nuclei have significantly less activity, and the microsomes and supernatant still less. In liver Ca^{++} and Mg^{++} also have dissimilar activating effects on ATP-ase of different cellular fractions: mitochondrial ATP-ase is activated by Mg^{++} but not activated by Ca^{++}, i.e., reacts to these metal ions in the same way as does the mitochondrial ATP-ase from brain. ATP-ase from liver nuclei and microsomes in activated by Mg^{++} and Ca^{++} just as is ATP-ase from brain nuclei and microsomes. However, the degree of activation of microsomal ATP-ase by these ions is not the same in brain and in liver.

Thus one must conclude that different cellular fractions of liver, of different cellular structures of brain, contain ATP-ase with different properties; and at the same time the properties of ATP-ases in corresponding cellular fraction of brain and liver are not the same.

SUMMARY

By means of the technique of differential centrifugation we obtained intracellular fractions of rabbit cerebral tissue: nuclei, mitochondria, microsomes, and the soluble (supernatant) fraction. Of the four cellular fractions, the mitochondria stand first in protein content (Table 1); the soluble fraction contains nearly as much protein; the nuclei have almost three time less protein; and the microsomes contain still less protein.

Adenosine triphosphatase occurs in all intracellular fractions of cerebrum: in nuclei, mitochondria, microsomes, and the soluble fraction. The most active ATP-ase is in the mitochondrial fraction; it is less active in the microsomal and nuclear fractions, in which its activity is almost the same. ATP-ase activity is very low in the solube fraction.

The ions calcium and magnesium have dissimilar activating effects on the ATP-ase of separate cellular fractions of cerebral tissue. Ca^{++} does not activate ATP-ase of the mitochondria and the soluble fraction, but has an activating effect on ATP-ase from nuclei and especially microsomes. Mg^{++} activates ATP-ase from all three cellular structures of the cerebrum, but the greatest activating effect of magnesium is on microsomal ATP--ase.

The differences in the activating effects of the ions calcium and magnesium on ATP-ase of separate cellular fractions suggests the assumption that they contain ATP-ase with different properties.

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All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of this issue.

IN THE ALL-UNION BIOCHEMICAL SOCIETY

Translated from Biokhimiya Vol 26, No. 2,
pp. 391-392, February, 1961

In 1960 the All-Union Biochemical Stociety carried out much work on the organizational strengthening of the affiliates and division of the Society. At the present time it normally directs the work of 37 Republic Societies and city divisions which bring together 2300 member of the Society. It has also carried out the work of preparing for the V International Biochemical Congress which will be held in Moscow in August, 1961.

In the Azerbaidzhan Biochemical Society there have been 16 sessions of the Society and 6 sessions of the Council. There were presentations of 21 papers on general biochemistry, biochemistry of plants, biochemistry of animals, and medical chemistry. The plans and papers of the scientific workers were discussed. Members of the society conducted much work on oral and written propaganda for biochemical science. A continually active consultative group functioned to help young scientific workers. In the Armenian Biochemical Society there were 12 sessions of the Society with papers on the themes "Nervous Regulation of Endocrine Glands", "The Action of Chloroprene on Some Phases of Metabolism", etc. In the Byelo Russian Biochemical Society there were 17 sessions. Plans were drawn up and discussed for the development of biochemistry in the BSSR for 15 years, on the basis of which recommendations were made to the Plenum of the Central Council of the All-Union Biochemical Society. Aid was given by the members of the Society in the organization of a department of general chemistry and biochemistry in the Grodnensk Medical Institute. A number of members of the Soceity took part in the popular scientific lectures planned by the Society for the spread of scientific and political knowledge in plants and institutes.

In the Gorkii division there were 33 scientific reports. Of these, 23 were experimental and 10 reviews and reports of meetings. There were 11 sessions for hearing and discussion of scientific addresses. Beside the scientific reports the sessions of the Society heard information and discussion of organization questions, plans of work, reports of the divisional Council, and reports of the Auditing Committee. In the Georgian Biochemical Society there were 10 general meetings and 10 sessions at which scientific papers and organizational questions were considered. There was a conference on "Biochemistry and Protein Metabolism". In Irkutsk the biochemical division held 6 sessions with discussion of scientific papers and plans of work. In the Kazakhstan Biochemical Society there were 6 sessions with discussion of papers. Preparations were made for holding the 2nd Kazakhstan Biochemical Conference. In the Kirgiz Biochemical Society there were regular sessions with discussions of scientific papers and preparations were made for discussions of scientific papers and preparations were made for the II Congress of Biochemists of Central Asia.

In the Krasnodar division there were sessions with discussions of scientific papers, consultations were held at various collective farms, and there were seminars on questions of fermentation regimes. In the Latvian Biochemical Society there were several sessions at which scientific papers and reports from member of the Society were heard. At these sessions there were also discussions and organizational problems. The council of the Society organized for the members courses in the study of the English language.

The Leningrad division held 6 sessions of the divisional council to consider organizational questions. There were 16 sessions with discussion of scientific papers and 4 sessions with other scientific societies. The themes of the papers and programs of the Society were approved at sessions of the Council. The work of the members of the Society on popularization of scientific knowledge was carried out at places of work, and through the Society by distribution of political and scientific information. A scientific conference was held, devoted to phosphorylation and function. In the Moldavian Biochemical Society there were sessions at which 19 scientific papers were

heard. The members of the Society gave scientific advice on question of method, chromatographic analysis on columns and paper, paper electrophoresis, purification of medicinal preparations, etc.

In the Moscow Biochemical Society there were monthly scientific sessions by the members of the Society and sessions of the Society Council. A conference was held on cyto- and histochemistry and work was carried out in preparation for a conference on carbohydrates. The Maritime Biochemical Division held several sessions with discussion of scientific and organizational questions. In the Rostov-on-Don division there were 10 sessions. The sessions were devoted to discussions of experimental investigations by members of the biochemistry departments of the University, Medical Institute, Antiplague Institute, and other. Members of the Society held systematic consultations with workers in the different plants in the city of Rostov-on-Don concerning methodological questions, and questions of setting up biochemical studies. There were 25 lectures for workers and employees of the plants and for the people of the city to popularize contemporary biochemistry. The division organized a biochemical section at the Conference of the Society of Physiologists of the South-East RSFSR, which was held in Rostov-on-Don in June, 1960. In the Sverdlovsk division there were 9 sessions including two with the Department of Biochemistry of the Sverdlovsk Medical Institute and one jointly with the Society of Physiologists. There were 19 papers and reports.

In the Tadzhikistan Biochemical Society there were regular sessions with discussions of scientific papers. In the Uzbekistan Biochemical Society there were a number of sessions in which organizational questions were decided and scientific addresses were heard. The council of the Society carried out measures for the intensification of biochemical investigations in the Republic, determining the main directions of research work in biochemistry, the improvement of laboratory equipment for biochemistry in structure, apparatus, etc.

The Ukrainian Biochemical Society is made up of 7 divisions: Kiev, Kharkov, Odessa, Dnepropetrovsk, Chernovits, Lvov, and Stalinsk. In all these divisions there were regular scientific sessions with discussions of the Society took an active part in the distribution of political and scientific information both by organization and by giving lectures. The members of the Society gave more than 200 lectures in plants and collective farms. They gave much consultative help on the basis of present day methods of biochemical investigation and on different phases of biochemical problems. The members of the Society gave a number of address for the citizens of the cities on the role and significance of biochemistry. In the Chelyabinsk division during this period there were 7 sessions at which there were addresses on theoretical questions of biological chemistry and demonstrations of methods of biological investigation. In the Esthonian Biochemical Society (Tartu and Tallin biochemical divisions) there were sessions for hearing and discussion scientific papers. The Tallin division was organized at the end of 1960. In the Yakutsk Biochemical Society there were three general meetings and 4 scientific papers were heard.

The Astrakhan, Voronezh, Crimean, Kursk, Litovsk, Smolensk, and Stalingrad divisions were organized at the end of 1960.

In all the republic societies and divisions the members of the Society received cards used for collection of entrance and member fees for the work of attracting new members. Joint conferences were held for the biochemical societies of the Baltic countries (Byelorussia, Latvia, Lithuania, Esthonia) devoted to the twenty years of the Latvian, Lithuanian, and Esthonian Soviet Socialist Republics. The program discussed measures for increasing biochemical research in the USSR.

The following commissions were at work: on preparing an All-Union conference on training specialists in biochemistry (president, Prof. S. R. Mardashov); on the question of biochemical nomenclature (president, Prof. A. E. Braumshtein); on publication (president, Prof. B. N. Stepanenko).

E. V. Budnitskaya

Soviet Journals Available in Cover-to-Cover Translation

ABBREVIATION	RUSSIAN TITLE	TITLE OF TRANSLATION	PUBLISHER	TRANSLATION BEGAN Vol.	Issue	Year
AÉ	Atomnaya énergiya	Soviet Journal of Atomic Energy	Consultants Bureau	1	1	1956
Akust. zh.	Akusticheskii zhurnal	Soviet Physics – Acoustics	American Institute of Physics	1	1	1955
	Antibiotiki	Antibiotics	Consultants Bureau	4	1	1959
Astr(on). zh(urn).	Astronomicheskii zhurnal	Soviet Astronomy–AJ	American Institute of Physics	34	1	1957
Avto(mat). svarka	Avtomaticheskaya svarka	Automatic Welding	British Welding Research Association (London)		1	1959
	Avtomatika i Telemekhanika	Automation and Remote Control	Instrument Society of America	27	1	1956
	Biofizika	Biophysics	National Institutes of Health*		1	1957
	Biokhimiya	Biochemistry	Consultants Bureau	21	1	1956
Byull. éksp(erim). biol. i med.	Byulleten' éksperimental'noi biologii i meditsiny	Bulletin of Experimental Biology and Medicine	Consultants Bureau	41	1	1959
DAN (SSSR) / Dokl(ady) AN SSSR	Doklady Akademii Nauk SSSR	The translation of this journal is published in sections, as follows:				
		Life Sciences: Doklady Biochemistry Section	American Institute of Biological Sciences	106	1	1956
		Life Sciences: Doklady Biological Sciences Sections (Includes: Anatomy, biophysics, cytology, ecology, embryology, endocrinology, evolutionary morphology, genetics, histology, hydrobiology microbiology, morphology, parasitology, physiology, zoology sections)	American Institute of Biological Sciences	112	1	1957
		Life Sciences: Doklady Botanical Sciences Sections (Includes: Botany, phytopathology, plant anatomy, plant ecology, plant embryology, plant physiology, plant morphology sections)	American Institute of Biological Sciences	112	1	1957
		Chemical Sciences: Proceedings of the Academy of Sciences of the USSR, Section: Chemical Technology	Consultants Bureau	106	1	1956
		Chemical Sciences: Proceedings of the Academy of Sciences of the USSR, Section: Chemistry	Consultants Bureau	106	1	1956
		Chemical Sciences: Proceedings of the Academy of Sciences of the USSR, Section: Physical Chemistry	Consultants Bureau	112	1	1957
		Earth Sciences: Doklady Earth Sciences Sections (Includes: Geochemistry, geology, geophysics, hydrogeology, mineralogy, paleontology, petrography, permafrost sections)	American Geological Institute	124	1	1959
		Earth Sciences: Proceedings of the Academy of Sciences of the USSR, Section: Geochemistry	Consultants Bureau	106-123	1-6	1957-1958
		Earth Sciences: Proceedings of the Academy of Sciences of the USSR, Sections: Geology	Consultants Bureau	106-123	1-6	1957-1958
		Mathematics: Doklady Soviet Mathematics	The American Mathematics Society	131	1	1961
		Physics: Soviet Physics–Doklady (Includes: Aerodynamics, astronomy, crystallography, cybernetics and control theory, electrical engineering, energetics, fluid mechanics, heat engineering, hydraulics, mathematical physics, mechanics, physics, technical physics, theory of elasticity sections)	American Institute of Physics	106	1	1956
		Physics: Proceedings of the Academy of Sciences of the USSR, Applied Physics Sections (does not include mathematical physics or physics sections)	Consultants Bureau	106-117	1	1956-1957
Derevoobrabat. prom-st'.	Derevoobrabatyvayushchaya promyshlennost'	Wood Processing Industry	Timber Development Association (London)		9	1959
	Élecktrosvyaz	Telecommunications	Massachusetts Institute of Technology*		1	1957
Éntom(ol). oboz(renie)	Éntomologicheskoe obozrenie	Entomological Review	American Institute of Biological Sciences	38	1	1959
Farmakol. (i) toksikol(ogiya)	Farmakologiya i toksikologiya	Pharmacology and Toxicology	Consultants Bureau	20	1	1957
FMM	Fizika metallov i metallovedenie	Physics of Metals and Metallography	Acta Metallurgica*	5	1	1957
Fiziol. zhurn. SSSR (im. Sechenova)	Fiziologicheskii zhurnal im. I. M. Sechenova	Sechenov Physiological Journal USSR	National Institutes of Health*		1	1957
Fiziol(ogiya) rast.	Fiziologiya rastenii	Plant Physiology	American Institute of Biological Sciences	4	1	1957
	Geokhimiya	Geochemistry	The Geochemical Society		1	1958
FTT	Fizika tverdogo tela	Soviet Physics–Solid State	American Institute of Physics	1	1	1959
Izmerit. tekh(nika)	Izmeritel'naya tekhnika	Measurement Techniques	Instrument Society of America		1	1959
Izv. AN SSSR, O(td). Kh(im). N(auk)	Izvestiya Akademii Nauk SSSR: Otdelenie khimicheskikh nauk	Bulletin of the Academy of Sciences of the USSR: Division of Chemical Sciences	Consultants Bureau		1	1952

continued

Izv. AN SSSR, O(td). T(ekhn). N(auk): Met(all). i top.	(see Met. i top.)					
Izv. AN SSSR Ser. fiz(ich).	Izvestiya Akademii Nauk SSSR: Seriya fizicheskaya	Bulletin of the Academy of Sciences of the USSR: Physical Series	Columbia Technical Translations		1	1954
Izv. AN SSSR Ser. geofiz.	Izvestiya Akademii Nauk SSSR: Seriya geofizicheskaya	Bulletin (Izvestiya) of the Academy of Sciences USSR: Geophysics Series	American Geophysical Union		1	1954
Izv. AN SSSR Ser. geol.	Izvestiya Akademii Nauk SSSR: Seriya geologicheskaya	Izvestiya of the Academy of Sciences of the USSR: Geologic Series	American Geological Institute		1	1958
Kauch. i rez.	Kauchuk i rezina	Soviet Rubber Technology	Research Association of British Rubber Manufacturers	18	1	1959
	Kinetika i kataliz	Kinetics and Catalysis	Consultants Bureau	1	3	1960
	Koks i khimiya	Coke and Chemistry USSR	Coal Tar Research Association (Leeds, England)		1	1958
Kolloidn. zh(urn).	Kolloidnyi zhurnal	Colloid Journal	Consultants Bureau	14	1	1952
	Kristallografiya	Soviet Physics – Crystallography	American Institute of Physics	2	1	1957
Metalov. i term. obrabot. metal.	Metallovedenie i termicheskaya obrabotka metallov	Metal Science and Heat Treatment of Metals	Acta Metallurgica	6	1	1958
	Metallurg	Metallurgist	Acta Metallurgica		1	1957
Met. i top.	Metallurgiya i topliva	Russian Metallurgy and Fuels	Eagle Technical Publications		1	1960
Mikrobiol.	Mikrobiologiya	Microbiology	American Institute of Biological Sciences	26	1	1957
OS	Optika i spektroskopiya	Optics and Spectroscopy	American Institute of Physics		6	1959
	Pochvovedenie	Soviet Soil Science	American Institute of Biological Sciences		1	1958
	Priborostroenie	Instrument Construction	British Scientific Instrument Research Association		1	1959
Pribory i tekhn. éks(perimenta)	Pribory i tekhnika éksperimenta	Instruments and Experimental Techniques	Instrument Society of America		1	1957
Prikl. matem. i mekh.	Prikladnaya matematika i mekhanika	Applied Mathematics and Mechanics	American Society of Mechanical Engineers		1	1958
PTÉ	(see Pribory i tekhn. éks.)					
	Problemy Severa	Problems of the North	National Research Council of Canada			
Radiotekh.	Radiotekhnika	Radio Engineering	Massachusetts Institute of Technology*	12	1	1957
Radiotekh. i élektronika	Radiotekhnika i élektronika	Radio Engineering and Electronics	Massachusetts Institute of Technology*	2	1	1957
	Stanki i instrument	Machines and Tooling	Production Engineering Research Assoc.		1	1959
	Stal'	Stal (In English)	Iron and Steel Institute		1	1959
Stek. i keram.	Steklo i keramika	Glass and Ceramics	Consultants Bureau	13	1	1956
Svaroch. proiz-vo	Svarochnoe proizvodstvo	Welding Production	British Welding Research Association		4	1959
Teor. veroyat. i prim.	Teoriya veroyatnostei i ee primenenie	Theory of Probability and Its Applications	Society for Industrial and Applied Mathematics		1	1956
Tsvet. Metally	Tsvetnye metally	Nonferrous Metals	Primary Sources		1	1960
UFN	Uspekhi fizicheskikh Nauk	Soviet Physics – Uspekhi (partial translation)	American Institute of Physics	66	1	1958
UKh	Uspekhi khimii	Russian Chemical Reviews	The Chemical Society (London)		1	1960
UMN	Uspekhi matematicheskikh nauk	Russian Mathematical Surveys	London Mathematical Society	15	1	1960
Usp. fiz. nauk	(see UFN)					
Usp. khim(ii)	(see UKh)					
Usp. matem. nauk	(see UMN)					
Usp. sovr. biol.	Uspekhi sovremennoi biologii	Russian Review of Biology	Oliver and Boyd		48	1959
Vest. mashinostroeniya	Vestnik mashinostroeniya	Russian Engineering Journal	Production Engineering Research Assoc.		4	1959
Vop. gem. i per. krovi	Voprosy gematologii i perelivaniya krovi	Problems of Hematology and Blood Transfusion	National Institutes of Health*		1	1957
Vop. onk.	Voprosy onkologii	Problems of Oncology	National Institutes of Health*		1	1957
Vop. virusol.	Voprosy virusologii	Problems of Virology	National Institutes of Health*		1	1957
Zav(odsk). lab(oratoriya)	Zavodskaya laboratoriya	Industrial Laboratory	Instrument Society of America	25	1	1959
ZhAKh Zh. anal(it). khimii	Zhurnal analiticheskoi khimii	Journal of Analytical Chemistry USSR	Consultants Bureau	7	1	1952
ZhETF Zh. éksperim. i teor. fiz.	Zhurnal éksperimental'noi i theoreticheskoi fiziki	Soviet Physics–JETP	American Institute of Physics	28	1	1955
ZhFKh Zh. fiz. khimii	Zhurnal fizicheskoi khimii	Russian Journal of Physical Chemistry	The Chemical Society (London)		7	1959
ZhMÉI Zh(urn). mikrobiol. épidemiol. i immunobiol.	Zhurnal mikrobiologii, épidemiologii i immunobiologii	Journal of Microbiology, Epidemiology and Immunobiology	National Institutes of Health*		1	1957
ZhNKh Zh(urn). neorgan(ich). khim(ii)	Zhurnal neorganicheskoi khimii	The Russian Journal of Inorganic Chemistry	The Chemical Society (London)		1	1959
ZhOKh Zh(urn). obshch(ei) khimii	Zhurnal obshchei khimii	Journal of General Chemistry USSR	Consultants Bureau	19	1	1949
ZhPKh Zh(urn). prikl. khimii	Zhurnal prikladnoi khimli	Journal of Applied Chemistry USSR	Consultants Bureau	23	1	1950
ZhSKh Zh(urn). strukt. khimii	Zhurnal strukturnoi khimii	Journal of Structural Chemistry	Consultants Bureau	1	1	1960
ZhTF Zh(urn). tekhn. fiz.	Zhurnal teknicheskoi fiziki	Soviet Physics–Technical Physics	American Institute of Physics	26	1	1956
Zh(urn). vyssh. nervn. deyat. (im. Pavlova)	Zhurnal vysshei nervnoi deyatel'nosti (im. I. P. Pavlova)	Pavlov Journal of Higher Nervous Activity	National Institutes of Health*		1	1958

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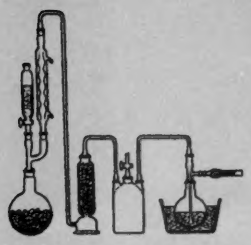

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